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*OBSERVATIONS ON THE STRUCTURE OF DEOXYRIBONUCLEOPROTEIN GELS**

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Communicated by Berwind P. Kaufmann, June 29, 1956

Deoxyribonucleic acid (DNA) has frequently been called a remarkably stable biological substance, reflecting the drastic methods required for its hydrolysis,[1] and its low rate of metabolic turnover.[2] With respect to its biological effects, however, the highly polymerized DNA molecule is very sensitive to relatively mild physical treatments.[3] Indications that DNA in combination with protein is unstable with respect to its molecular integrity as a highly polymerized structure have been derived from work on deoxyribonucleoproteins.[4, 5] The present report extends those investigations by analysis of the effects of heat, 1.0 *M* NaCl, trypsin, and urea on the macromolecular structure and organization of deoxyribonucleoproteins. The studies, combined with experiments on a model nucleoprotein system, have given additional indications of the complexity and sensitivity of these components of cell nuclei.

Materials and Methods.—The deoxyribonucleoprotein used in this work was obtained from isolated calf thymus nuclei. Frozen calf thymus was chopped into pieces of suitable size and comminuted in an electric meat-grinder. The ground tissue was suspended in cold 0.14 *M* NaCl, stirred vigorously for 5 minutes, and filtered rapidly through one layer of coarse (No. 10) cheesecloth and then through one layer of fine (No. 80) cheesecloth. The filtrate was centrifuged in the cold at $100 \times g$ for 1 minute in order to separate nuclei from cytoplasmic materials. The supernatant fluid was decanted and the nuclei resuspended in cold 0.14 *M* NaCl and centrifuged as before. The saline washes were repeated three to five times and were followed by two or three washings in distilled water. The nuclear material is then in the form of a rigid gel, and the preparation of the nucleoprotein was completed simply by allowing the gel to take up more water. In these experiments the gels were prepared so as to contain approximately 2 mg. of deoxyribonucleoprotein per milliliter.

Dounce and his co-workers[6] have recently investigated methods of isolating "gellable" nuclei. The essential and common features of their work and of the present study would appear to be a restrained violence adequate for cell disruption but not producing extensive destruction of nuclei or mitochondria, and the fairly rapid separation of nuclei from cytoplasmic materials. The mechanisms of gel formation by nuclei are not yet clear. Washing in water and the consequent re-

moval of electrolytes may permit a greater expression of the repulsive forces of similarly charged portions of the nucleoprotein molecules.

The DNA was prepared from frozen salmon testes (New England Fish Company, Seattle, Washington) according to the method of Emannuel and Chaikoff,[7] except that the DNA was recovered by fractional precipitation with alcohol and the adsorbed NaCl was removed by washing in alcohol. The bovine serum albumin was a crystalline preparation purchased from Armour and Company; the trypsin was a salt-free preparation, crystallized by the method of McDonald and Kunitz.[8]

Viscosity measurements were made in Cannon-Fenske type Ostwald viscometers, maintained, unless otherwise stated, in a water bath at 25° C.

Results.—Gels made by suspending calf thymus nuclei in water exhibit a marked spontaneous loss of structural rigidity as measured by flow time through a capillary viscometer. This phenomenon is markedly temperature dependent, as shown in Figure 1. The measurements at 25° and 37° C. were made directly. The data of the 5° C. curve represent initial values of serial samples from a stock preparation that had been kept at 5° C. but measured at 25° C. after a 5- to 10-minute period for temperature equilibration. The thermolabile instability of the nucleoprotein gel is apparent.

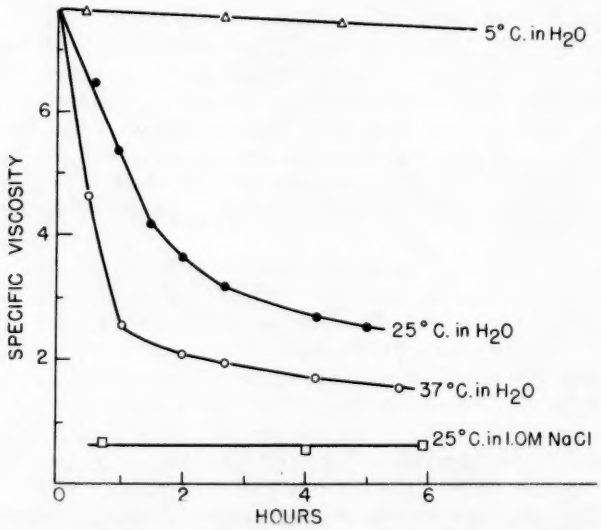

FIG. 1.—The effects of temperature and 1.0 *M* NaCl on the specific viscosity of deoxyribonucleoprotein gels.

DNA-containing cell fractions are commonly found to increase in viscosity when placed in 1 *M* NaCl.[9] A quite different behavior is found when an aqueous nuclear gel is brought to this salt concentration by the addition of 5 *M* NaCl. As shown in Figure 1, the viscosity is drastically reduced, by approximately tenfold, then remains constant over a period of several hours at 25° C. It has been shown that DNA and protein are dissociated in 1.0 *M* NaCl;[10] thus it would appear that a major

portion of the viscosity of the aqueous nucleoprotein gels is due to factors other than the asymmetry of the DNA molecule.

This conclusion was further supported by observations on the effects of storage at 4° and 25° C. on deoxyribonucleoprotein gels Freshly prepared gels were stored for 4 hours at these temperatures, and the viscosity of each preparation was measured in water and in 1.0 *M* NaCl. The results are presented in Table 1, and

TABLE 1

EFFECT OF STORAGE ON VISCOSITY OF DEOXYRIBONUCLEOPROTEIN

	Specific Viscosity of		
	Original Sample (1.2 mg. DNA per ml.)	Samples Aged 4 hours at 2°–5° C.	25° C.
Viscosity measured in water	120.0	. . .	35.0
Viscosity measured in 1.0 *M* NaCl	2.65	2.61	1.88

show that in water the gel has undergone a considerable degradation. In the strong salt solution the measurement is essentially dependent on the molecular asymmetry of the DNA, and there has apparently been only a small amount of degradation of the nucleic acid. These results further suggest that the observed gel structure is determined by interparticulate interactions involving protein.

Further investigations of the role of protein in the formation of these nuclear gels were undertaken. Addition of an inert protein, crystalline bovine serum albumin, to nucleoprotein gels produces an increase in the viscosity of the gel, as shown in Figure 2. Such a result would be expected if a major determinant of the gel structure involved secondary molecular interactions between protein molecules. On the other hand, the addition of the proteolytic enzyme, trypsin, produces a marked decrease in the viscosity of the gel. As seen in Figure 2, the effect is approximately proportional to the amount of trypsin added and is presumably dependent on its proteolytic activity. Additional evidence of the importance of protein in the gel structures being studied comes from the observation that the structural rigidity of the nucleoprotein gel is completely abolished by 6.6 *M* urea.[11]

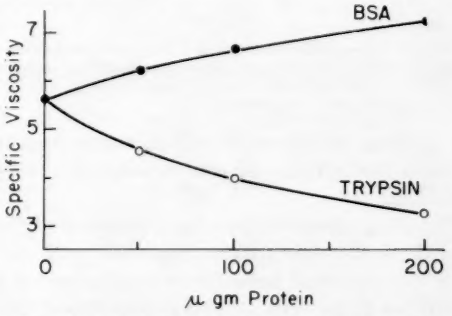

FIG. 2.– Addition of bovine serum albumin (BSA) and trypsin to deoxyribonucleoprotein gels. For each point the indicated amount of protein was added to an aliquot of nucleoprotein; the samples were mixed and held in the viscometers at 25° C. The specific viscosity shown was measured after 2 hours. Other experiments by the author (see Kaufmann, McDonald, and Bernstein, *Ann. N.Y. Acad. Sci.*, **59**, 553, 1955) have shown that the curves relating the trypsin-induced decrease in specific viscosity to time are similar to the 25° C. curve of Figure 1 but are displaced in the vertical dimension.

The data that have been presented are all consistent with the suggestion that the structure of the deoxyribonucleoprotein gel is largely determined by the protein moiety and its capacity for the formation of intermolecular associations.

To further clarify the individuality of deoxyribonucleoproteins, it was considered

desirable to study synthetic systems of nucleic acid and protein. As one of the main naturally occurring proteins associated with DNA, histone was the material of choice. However, the interaction of DNA and histone was found to produce an insoluble precipitate. A suitable system was achieved by mixing aqueous solutions of DNA and bovine serum albumin. Establishment of the complex was indicated by studies of the absorption spectra of the various substances,[12] as well as by viscosity data, which are shown for a typical experiment in Figure 3. The viscosity of the albumin is considerably lower than that of the DNA, but both are quite stable over periods of several hours at 25° C. The mixture of DNA and albumin has a viscosity significantly higher than the sum of the two components;[13] furthermore, the stability of the pure substances is replaced by the instability that has previously been found to be characteristic of nucleoproteins.

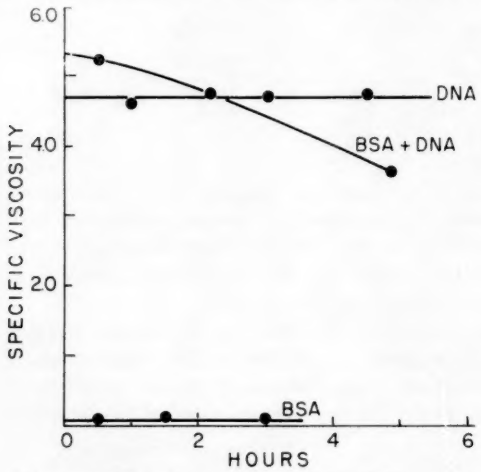

FIG. 3.—The specific viscosity of DNA, of BSA (bovine serum albumin), and of a synthetic nucleoprotein made by mixing DNA and BSA. Final concentrations are all 1.0 mg/ml, for each component.

Discussion.—An earlier report described the effects of X-rays on aqueous nucleoprotein gels.[11] Low-level irradiation accelerated the spontaneous degradation of the gel structure. It was shown that the irradiation had no effect on the polymer length of the DNA, and it was concluded that the irradiation influenced the capacity of the nucleoprotein molecule to form interparticulate associations.

The data presented here have emphasized the importance of the protein component of the deoxyribonucleoprotein molecule. DNA is generally considered to be the material basis for the maintenance and transmission of the "genetic record" of living cells. Current concepts of the structure of the DNA molecule[14] present certain difficulties with regard to an understanding of genetic mechanisms in terms of the rigidity of these models. Problems pertaining to the genetic requirements of replication, crossing over, inversions, translocations, and related phenomena are difficult to reconcile with the proposed models. The behavioral modifications imposed on DNA by combination with protein would appear to offer valuable assistance in explaining these phenomena.

The available evidence[5, 9, 15] suggests that the linkage of protein to nucleic acid is accomplished by way of salt linkages between oppositely charged groups. An electrostatic bond is the most obvious, but in many ways the least satisfactory, possibility. Covalent or co-ordinate linkages are possible, and these will introduce an instability into the pattern of internucleotide linkages. Assuming a reasonable frequency of these protein linkages, they will tend to preserve the nucleic acid–

protein association, and any breakdown or modification of the molecule will produce shorter *nucleoprotein* fragments.[4, 5] Obviously, such behavioral characteristics would be of considerable assistance in reconciling the genetic and physicochemical requirements of the hereditary substance.

From a different standpoint, a chromosome may be considered as a close-packed arrangement for the transmission of material from cell to cell. There is general agreement that any functional state of chromosomes must relate to the more dispersed state of the material in the interphase nucleus. From the data presented here, it is possible that the transition from the dispersed functional state to the inert but highly coiled and otherwise organized form of the mitotic chromosome can be accomplished by manipulation of the intracellular ionic charge level.

Summary.—Deoxyribonucleoprotein gels were made by washing isolated calf thymus nuclei in distilled water. Measurements on the flow times of such nucleoprotein gels through capillary viscometers were made.

Deoxyribonucleoprotein gels are thermolabile and exhibit a marked spontaneous instability. The high anomalous viscosity of the gels is abolished by 1.0 *M* NaCl and by 6.6 *M* urea. The viscosity of a deoxyribonucleoprotein gel is increased by the addition of serum albumin and decreased by the addition of trypsin.

The high anomalous viscosity of the deoxyribonucleoprotein gels is not attributable solely to the presence of highly polymerized DNA. The gel structure is determined largely by intermolecular associational bonds in which proteins are primary participants.

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The author wishes to express his thanks to Dr. Margaret McDonald for her counsel during the course of these investigations.

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[12] Other workers have also noted that the absorption of synthetic nucleoproteins is not additive (E. R. Blout and A. Asadourian, *Biochim. et biophys. acta*, **13**, 151, 1954: F. B. Seibert, *Discussions Faraday Soc.*, **13**, 251, 1953).

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*THE EFFECT OF LIPOIC ACID ON RECOVERY IN INDUCED LYSOGENIC ORGANISMS**

By Ernest Borek,† Joyce Rockenbach, and Ann Ryan

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Communicated by David Rittenberg, June 6, 1956

In some strains of lysogenic micro-organisms the metastable balance between prophage and host can readily be disrupted and the proliferation of bacteriophage initiated by small doses of ultraviolet irradiation.[1]

In studies of the metabolism of such "induced" lysogenic cells we have observed that enrichment of the culture medium with acetate during the period of phage development can moderate the lysogenic response. Addition to *Escherichia coli* K12 of 5 mg. of acetate per milliliter 1 minute after irradiation can increase by as much as tenfold the number of cells which survive as colony-formers; a somewhat diminished effect is still measurable when the acetate is added 15 minutes after the irradiation.

Reduced CoA[2] when added with or without GSH had no effect on the induced cultures—the number of cells which could survive to produce colonies did not differ from those in identical controls—nor did the addition of DPN, TPN, ATP, or TPP. However, when CoA was added together with acetate, the increment in the cells capable of giving rise to colonies was greater than with acetate alone (Table 1). CoA was unique among the cofactors tested in producing this potentiating

TABLE 1*

Effect of Acetate and CoA on Survival of Induced *E. coli* K12

Irradiation (Seconds)	Adjuvant	Time of Addition after Irradiation (Minutes)	Cell Count before Irradiation ($\times 10^{-8}$)	Surviving Cell Count ($\times 10^{-2}$)
250	.....	..	1.6	3.5
250	Acetate, 5 mg/ml	1	1.6	20
250	Acetate, 5 mg/ml; CoA, 0.5 mg/ml	1	1.6	140
				($\times 10^{-6}$)
120	.....	..	3.0	5
120	Acetate, 5 mg/ml	1	3.0	31
120	Acetate, 5 mg/ml	10	3.0	16
120	Acetate, 5 mg/ml	15	3.0	14

* *E. coli* K12 in logarithmic growth phase in glucose-salts medium was exposed to irradiation for the periods indicated. At intervals after the irradiation, adjuvants were added to identical aliquots and the cultures were incubated in the dark for 50 minutes. The cells—and adjuvants—were then diluted by serial dilution, and aliquots were plated on nutrient agar. For experimental details see E. Borek, A. Ryan, and J. Rockenbach, *J. Bacteriol.*, **69**, 460, 1955.

effect. Under identical conditions acetate plus CoA consistently had a slightly depressing effect on the survival of irradiated nonlysogenic organisms, *E. coli* B and *E. coli* B/r (see Table 3).

Pyruvate plus lipoic acid, a cofactor of its dissimilation to acetate,[3] was more effective than acetate plus CoA in increasing the fraction of surviving cells capable of giving rise to colonies. Moreover, lipoic acid alone was found to be the most potent single adjuvant for such restoration effect (see Exp. 1, Table 2). Lipoic acid, as acetate, was effective when added as late as 15 minutes after the irradiation.

Lipoic acid alone or augmented with pyruvate had a slightly depressing effect on the survival of irradiated *E. coli* B and B/r (Table 3).

TABLE 2*

EFFECT OF LIPOIC ACID ON SURVIVAL OF INDUCED *E. coli* K12

Irradiation (Seconds)	Adjuvant	Time of Addition after Irradiation (Minutes)	Cell Count before Irradiation ($\times 10^{-8}$)	Surviving Cell Count ($\times 10^{-5}$)	Plaque Count ($\times 10^{-8}$)
Experiment 1:					
120	.....	..	1.1	3.8	..
120	Pyruvate, 5 mg/ml	1	1.1	28	..
120	Lipoic acid, 100 μg/ml	1	1.1	86	..
120	Pyruvate plus lipoic acid—amounts as above	1	1.1	140	..
Experiment 2:					
90	.....	..	1.7	58	1.6
90	Pyruvate plus lipoic acid—amounts as in Exp. 1	1	1.7	210	1.2

* Experimental conditions as in Table 1.

TABLE 3*

EFFECT OF ACETATE AND OF LIPOIC ACID ON SURVIVAL OF IRRADIATED *E. coli* B/r

Irradiation (Seconds)	Adjuvant	Time of Addition after UV (Minutes)	Cell Count before UV ($\times 10^{-8}$)	Surviving Cell Count ($\times 10^{-5}$)
250	.....	..	1.8	4
250	Acetate, 5 mg/ml	1	1.8	2.5
250	Lipoic acid, 100 μg/ml	1	1.8	2.3

* Experimental conditions as in Table 1.

It has been shown by Weigle and Delbrück[4] that in *E. coli* K12 nearly every cell can be induced by irradiation to become a plaque-former, provided that the irradiation administered is carefully controlled. With too small a dose of irradiation significant fractions of the cells survive to form colonies. With increasing doses of irradiation a level is reached where the number of plaques equals the number of cells before induction. However, if the irradiation is increased beyond this level, the number of plaques decreases exponentially. In such an overirradiated culture the origin of the colony-formers restored by lipoic acid is obscure. They could arise from the restoration of cells which would have given rise to plaques, or they could arise from the more heavily damaged cells which would have died without giving rise to phage. However, in cultures receiving the optimum dose of inducing irradiation—90 seconds in our experiments—where a complete tally of plaque-formers and colony-formers is possible, the origin of the cells restored by lipoic acid is unequivocally the fraction which would have yielded plaques. In such cultures reduction in the number of plaques is a concomitance of the increase in colony-formers (Exp. 2, Table 2).

Restoration of induced lysogenic cultures has been demonstrated heretofore by visible light[5] and by premature plating on agar after induction,[1, 4] but the mechanism or the operational site of such restorations is obscure. However, the finding that external reinforcement by a cofactor of pyruvate metabolism or by the end-products of that metabolism can partially restore a culture of induced lysogenic

organisms suggests that the system yielding acetate from pyruvate may be involved in the mechanism controlling the balance between prophage and host.

We could find no effect by the addition of lipoic acid, or any other of the adjuvants, on systems of virulent phase infection—by T1—of either lysogenic or nonlysogenic organisms as measured by efficiency of infection, survivors, and burst size, in one-step growth experiments.[6] This finding was not unexpected. In a system of an organism invaded by a virulent phage the host must cope not only with the invading DNA but with the toxic protein moiety as well. An induced lysogenic organism where there is no foreign protein involved could thus be expected to be more amenable to restoration during the early phase after induction. However, it may be relevant that Spizizen[7] has shown that *E. coli* grown on lactate or L-serine accumulates pyruvate after infection by phage T2 or T7, indicating that some alteration of pyruvate metabolism may be a concomitance of infection by virulent phage as well.

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[2] The following abbreviations are used: "CoA" for coenzyme A, "ATP" for adenosine triphosphate, "DPN" for diphosphopyridine nucleotide, "TPN" for triphosphopyridine nucleotide, "TPP" for thiamine pyrophosphate, and "GSH" for glutathione.

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*LIGHT-INDUCED PARAMAGNETISM IN CHLOROPLASTS**

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Communicated by E. U. Condon, July 28, 1956

I. *Introduction.*—Oxidation-reduction processes, which play a pervasive role in the chemistry of living cells, necessarily involve transfer of electrons. It has been proposed by Michaelis[1] and others that biochemical electron transfers occur singly rather than in pairs and that as a result free radicals (i.e., molecules possessing an unpaired electron) occur as intermediates. This proposal leads to the expectation that small steady-state concentrations of free radical intermediates ought to be found in metabolically active tissues. Evidence on the occurrence of free radicals during the course of metabolic processes is therefore of interest because it may be expected to enhance our understanding of the physical processes which govern biological oxidations.

In this regard, the possible role of free radicals in photosynthesis is of particular interest. Photosynthesis clearly involves oxidation-reduction steps in which free radicals might occur as intermediates. Also, it has been proposed that the light-induced excitation of chlorophyll results in the formation of a triplet state which mediates the transfer of light energy to the consequent chemical events of photosynthesis.[2] Lewis and Kasha[3] have shown that molecules in such a triplet state are biradicals in which two unpaired electrons occur and which are therefore paramagnetic.

It was discovered by Commoner, Townsend, and Pake[4] that free radicals can be detected in a wide array of biological systems by the paramagnetic resonance absorption technique. This method detects the absorption of microwave energy which results from the interaction between a variable external magnetic field and the magnetic moments of unpaired electrons present in the sample. In our earlier work, the design and sensitivity characteristics of the apparatus required that the samples tested be essentially free of liquid water. Consequently it was possible only to show that paramagnetic resonance absorptions due to free radicals occur in lyophilized powders prepared from various tissues in different states of metabolic activity. This procedure precluded detection of very labile forms of free radicals which might not survive the lyophilization process, and did not permit us to establish that free radicals were kinetically active in metabolic processes.

The recent construction of a highly sensitive apparatus capable of detecting the paramagnetic resonance absorption due to small amounts of free radicals in systems containing liquid water has now permitted investigations of active biochemical preparations. The present paper reports studies of the effect of light on the paramagnetic resonance absorption exhibited by suspensions of chloroplasts. Such preparations are known to carry out a number of the steps of photosynthesis.[5] We find that illumination of chloroplast preparations induces a large and very rapid formation of paramagnetic material, the properties of which are consistent with those of an intermediate in some phase of photosynthesis.

II. *Methods.*—Chloroplast preparations were obtained from leaves of greenhouse-grown plants of *Nicotiana tabacum* (variety White Burley). Blade tissue was gently macerated in an ice bath by hand in 0.05 *M* Tris buffer (pH 8) which was 0.88 *M* with respect to sucrose. Debris was removed by filtration through cheesecloth and by centrifugation for 5 minutes at 100 × *g*. The suspension was then centrifuged for 1 hour at 2500 × *g* and the supernatant discarded. The precipitate of chloroplasts was resuspended in 55 per cent sucrose and centrifuged for $^1/_2$ hour at 41,000 × *g*. In sucrose of this concentration, chloroplasts fail to sediment, while nuclei, mitochondria, and related particles are found in the pellet. The supernatant chloroplast fraction was removed and washed twice in pH 7.0 phosphate buffer which was 0.5 *M* with respect to sucrose and 0.003 *M* with respect to $NaHCO_3$. It was stored in the cold in this solution.

The chloroplast suspensions were transferred to flat glass cells (0.5 × 1 × 20 mm.) for resonance measurements. The apparatus employed for this purpose measures the absorption of 9,000-megacycle microwave energy incident upon the sample, as influenced by the variation of the strength of an externally applied magnetic field. The magnetic moment of an unpaired electron leads to absorption of 9,000-megacycle radiation at an external magnetic field of 3,300 oersteds. Incident

microwave power was generated by a klystron at a level of about 10 milliwatts. It was transmitted via a wave-guide system to a resonant cavity containing an opening to accommodate the sample tube, and the output power from the cavity was detected by a silicon diode. The external magnetic field was swept slowly through the range in which absorption was expected and, in addition, was varied, or modulated, sinusoidally over a smaller range of a fraction of the width of the absorption peak. The frequency of this modulation was 100 kilocycles per second. The signal from the silicon diode was amplified and fed into a phase-sensitive detector, which selects the signal (which is coherent with the modulation of the magnetic field) and rejects most of the noise (which is incoherent). The signal is recorded as the sweeping of the magnetic field proceeds, and the resulting record is a derivative of absorption with respect to magnetic field. The apparatus is in general similar to that used by others,[6] except for the use of the high modulation frequency of 100 kilocycles per second, which has added considerably to the sensitivity. This apparatus will permit paramagnetic resonance absorption measurements of aqueous samples about 0.04 ml. in volume. Its sensitivity is sufficient to detect paramagnetic resonance absorption due to the unpaired electrons of Mn^{++} ions in 0.04 ml. of an aqueous 10^{-5} M solution.

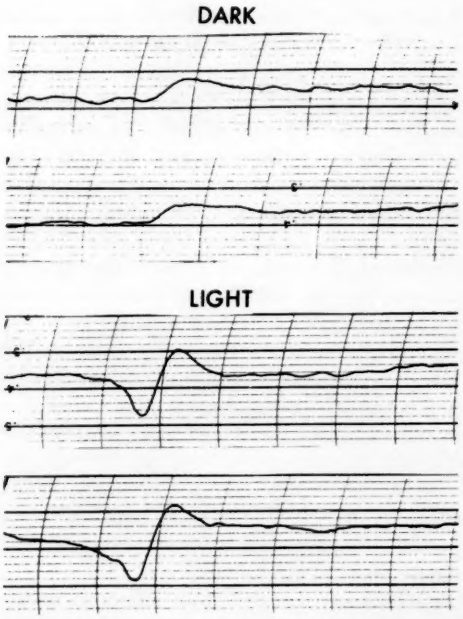

FIG. 1.—Paramagnetic resonance absorption of a chloroplast suspension in the dark (*upper curves*) and in the light (*lower curves*). Magnetic field increases toward the right along the abscissa. The ordinate is the meter deflection, representing the rate of change, with respect to field strength, of the absorption of 9,000-Mc/sec microwave energy by the sample, as the field strength is varied automatically. The curves thus represent derivatives of absorption curves. Each division along the abscissa represents approximately 20 gauss and 30 seconds. The half-width of the resonance is about 10 gauss.

The cavity end wall contains a hole about 2 mm. in diameter which permits illumination of the sample. The latter was held parallel to the wall about 3 cm. from it. Illumination was accomplished by means of a 50-c.p. automobile headlight lamp, using a lens to bring the filament image to a focus at the plane of the end wall. The sample temperature was about 35° C.

III. *Results.*—When chloroplast preparations are examined in the dark, small paramagnetic resonance absorptions are observed. The magnitudes of these vary with the age and treatment of the preparation. When such preparations are illuminated *in situ*, there occurs an obvious increase in paramagnetic resonance absorption. Results typical of this effect are shown in Figure 1, in which are re-

produced measurements of a darkened chloroplast suspension and of the same preparation under illumination. The relative number of unpaired electrons present can be roughly approximated from the maximum amplitude of the deflection in detector-meter value. Such a comparison of a number of tobacco chloroplast preparations indicates that the amplitude of paramagnetic resonance absorption of illuminated chloroplasts is about 3–6 times that exhibited by darkened chloroplasts.

Comparison of the chloroplast paramagnetic resonance absorption with that of a standard free radical (diphenyl picryl hydrazyl) shows that the resonances occur at the same external magnetic field, both being characterized by a g value of 2.002 ± 0.001. This indicates that the unpaired electrons present in illuminated chloroplasts have gyromagnetic properties that are not distinguishable from those of the great majority of the organic free radicals thus far investigated.[6] The width of the chloroplast absorption curve is about 10 gauss, and in this respect it is similar to resonances found in other biological materials.[4]

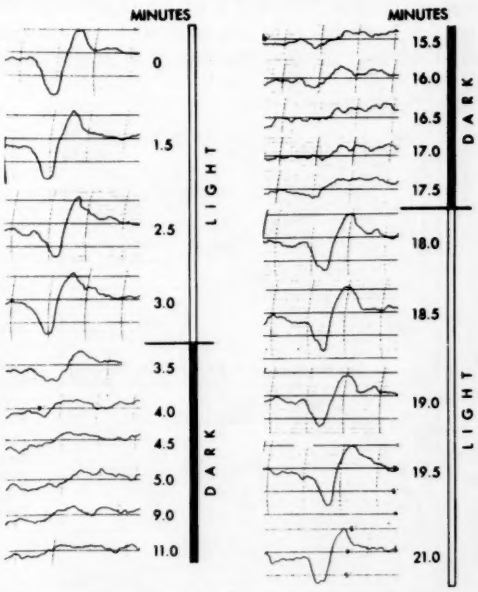

FIG. 2.—Paramagnetic resonance absorption of a chloroplast suspension exhibited at intervals during successive light, dark, and light periods. The times noted represent minutes elapsed after the first curve (*upper left corner*) was obtained, and refer to the center point of each absorption curve. Abscissa and ordinate are as in Fig. 1, except that each division along the abscissa represents approximately 16 gauss and 7.5 seconds.

The response of a chloroplast preparation to illumination is rapid, readily reversible, and recurrent. Figure 2 shows the results of a run in which the paramagnetic resonance absorption of a chloroplast preparation was determined by a series of rapid scans at intervals of about 30 seconds, during successive light, dark, and light periods. The results show that the light-induced paramagnetic resonance absorption decays to the dark value within a time of the order of 1 minute after illumination is halted. The light-induced paramagnetic resonance absorption attains a maximum value within 30 seconds after the onset of illumination. The effect of alternate light and dark can be observed in preparations for at least several hours after measurements are initiated and has been found in tobacco chloroplast preparations which have been stored in the cold for as long as 7 days.

The experiment reported in Figure 3 is a more detailed study of the time course of the appearance of paramagnetic resonance absorption on illumination and its decay in the dark. The paramagnetic resonance absorption of a chloroplast

preparation was determined, in several successive runs, at 1–5-second intervals after the light was turned on or off and was followed until a steady value for meter deflection was attained. Figure 3 shows that the maximum value of chloroplast paramagnetic resonance absorption is attained within about 20 seconds after the onset of illumination; the decay time is about 50–60 seconds. When the data of Figure 3 are displayed on a semilog plot, they approximate straight lines, indicating that the rates of appearance and decay of the resonance are exponential functions of time. The time constants for both processes have been calculated from these plots. For appearance of the resonances after onset of illumination the exponential time constant is about 12 seconds; this is equivalent to a half-time of about 8 seconds. The exponential time constant for decay of the resonance is about 45 seconds, and the half-time about 32 seconds.

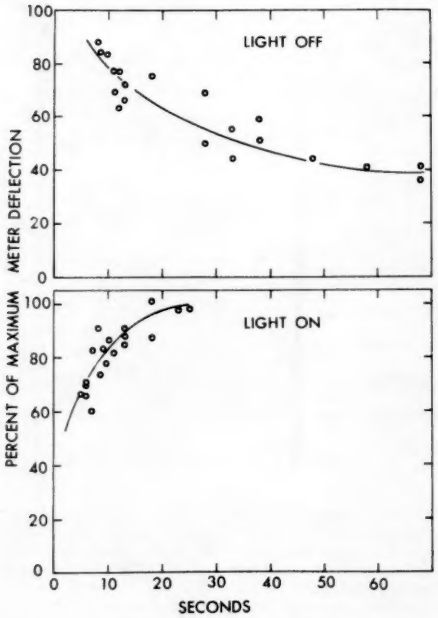

FIG. 3.—Intensity of paramagnetic resonance absorption exhibited by a chloroplast suspension at intervals after onset of light (*lower curve*) and cessation of light (*upper curve*). The points represent the results of 3 successive runs, made on the same preparation over a 2-hour period. In the upper curve, each point was determined after the sample had been illuminated for a 1-minute period. In the lower curve, each point was determined after the sample had been in the dark for a 3-minute period.

These results show that, when illuminated, chloroplasts produce unpaired electrons which are unstable and subject to a decay process with an exponential constant of about 45 seconds at 35° C. The amplitude of the paramagnetic resonance absorption in the light is evidently related to the steady-state concentration of unpaired electrons established by the opposed effects of their photochemical formation and the decay process.

Figure 4 shows that the light-induced paramagnetic resonance absorption cannot be due to a stable product of chloroplast photochemistry. Such a substance would be expected to accumulate during extended light periods. In contrast, Figure 4 shows that the paramagnetic resonance absorption exhibited by a chloroplast preparation remains unchanged when it is illuminated over a 23-minute period.

This confirms that the paramagnetic resonance absorption observed in illuminated chloroplasts is due to unpaired electrons in an unstable molecular configuration.

Figures 5 and 6 show the relationship between light intensity and the amplitude of chloroplast paramagnetic resonance absorption. It is evident that the relative number of unpaired electrons increases with light intensity. This relationship is expected from a system in which resonance amplitude reflects a steady state achieved by opposing light and decay processes. Figure 6 shows that the chloroplast paramagnetic resonance absorption is subject to light saturation. The occurrence of such a limit in the response of chloroplasts to light is characteristic of the photosynthetic activities of both chloroplasts and whole cells.

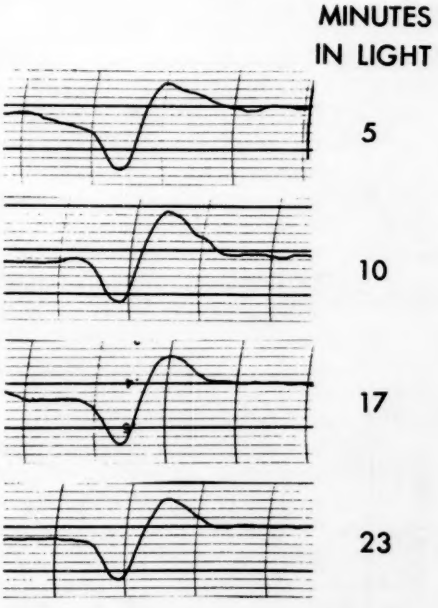

FIG. 4.—Paramagnetic resonance absorption of a chloroplast suspension at various intervals (5, 10, 17, and 23 minutes) after the onset of illumination. Ordinate and abscissa are as in Fig. 1, except that each division along the abscissa represents 30 seconds and about 16 gauss.

The wave-length dependence of chloroplast paramagnetic resonance absorption is comparable to that exhibited by the chloroplast processes related to photosynthesis. The latter occur in visible light extending from 400 to 700 mμ. This band of wave lengths is uniquely transmitted by a 1-cm. filter of a 2.5 per cent aqueous solution of $CuCl_2$. When a chloroplast preparation is illuminated by light from a tungsten filament after passing through such a filter, the usual increase in paramagnetic resonance absorption is observed.

Thus the foregoing evidence shows that chloroplasts respond to visible light by producing, very rapidly, unpaired electrons which are in an unstable configuration. Chloroplast paramagnetism is influenced by light intensity and wave length in a manner which parallels the effects of light on photosynthesis. This observation, together with the fact that the phenomenon is a property of the cellular component which is the seat of most of the phases of photosynthesis, suggests

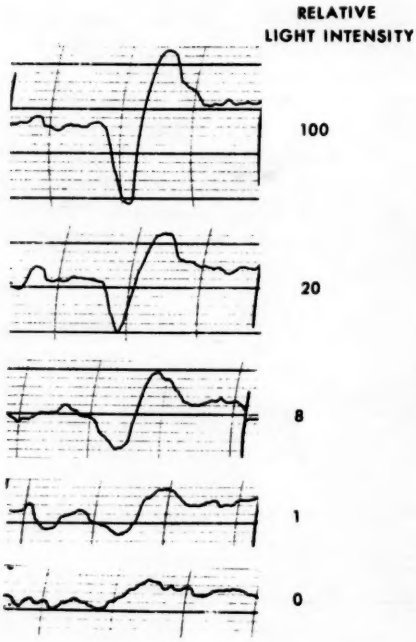

FIG. 5.—Paramagnetic resonance absorption of a chloroplast suspension illuminated at various light intensities. Light intensity was increased stepwise by means of a variable Polaroid filter, calibrated by photometer. Each curve was determined several minutes after the onset of illumination or, in the case of zero light, after about 10 minutes in the dark. Ordinate and abscissa are as in Fig. 1, except that each division along the abscissa represents 7.5 seconds and about 20 gauss.

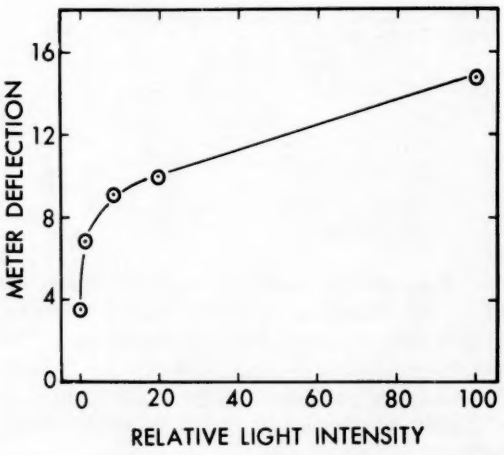

FIG. 6.—The relation between light intensity and the amplitude of paramagnetic resonance absorption of a chloroplast suspension. The points represent the mean values from 6–8 successive runs at each intensity and are determined from the same experiment described in Fig. 5.

that the unpaired electrons which occur in illuminated chloroplasts are associated with some steps in the photosynthetic process. This conclusion is given added support by the following considerations.

IV. *Discussion.*—The photochemical capabilities of chloroplast preparations, such as those employed in the foregoing experiments, are well known. Generally,

such preparations, when illuminated, will produce oxygen, apparently as a result of oxidative splitting of water, provided that suitable hydrogen acceptor is present. If only the natural hydrogen acceptors present in leaf extracts are available, chloroplasts will produce O_2 only if the ambient p_{O_2} is reduced to less than 2–4 mm. of Hg.[7, 8] Chloroplast preparations have been shown to reduce pyridine nucleotide coenzymes on illumination, and this process may be coupled enzymatically to CO_2 fixation.[9] In addition, it has been suggested that photochemical electron transport may also involve other reversibly oxidizable components, including flavine mononucleotide, vitamin K, thioctic acid, and ascorbic acid.[5, 10] Thus, on illumination, chloroplasts may, depending on their environment, be capable of (*a*) formation of stable photosynthetic products, including molecular O_2 and metabolites; (*b*) reduction of reversibly oxidizable metabolic electron carriers, e.g., DPN; (*c*) formation of the as yet undemonstrated chlorophyll biradical or some corresponding light-induced excited state. In what follows, we shall consider the possible contribution of each of these categories of effects to the paramagnetic resonance absorption of illuminated chloroplasts.

No metabolite product of photosynthesis is known to contain unpaired electrons; this group of substances cannot contribute to chloroplast paramagnetism. On the other hand, O_2 is paramagnetic and may exhibit paramagnetic resonance absorption which is considerably broader than those found in chloroplasts. However, it appears very unlikely that the results just described can be attributed to light-induced production of molecular O_2 or of a form of oxygen in equilibrium with molecular O_2. Since no hydrogen acceptors were added to our preparations, they were incapable of producing O_2 in an ambient p_{O_2} greater than 2–4 mm. of Hg.[7, 8] Our preparations were in equilibrium with air, and O_2 production is therefore precluded. Oxygen would be expected to accumulate during illumination. In contrast, the paramagnetic resonance of chloroplasts remains at a constant level over extended periods of time. The fact that the chloroplast paramagnetism decays rapidly in the dark is also evidence that it is not due to O_2. The chloroplast preparations are in equilibrium with the high ambient p_{O_2} of air, and there is no evidence for a chloroplast process capable of rapidly reducing this O_2 concentration.

On the other hand, the properties of chloroplast paramagnetism are consistent with the expected behavior of an intermediate in some stage of the light-induced electron transport which must occur in photosynthesis. As already noted, the paramagnetic component which we have detected is generated by light. The effective wave lengths are in the range 400–700 mμ. The process exhibits a distinct light-saturation effect. In these respects chloroplast paramagnetism parallels chloroplast photosynthesis. Furthermore, the existence of two classes of unstable paramagnetic components has been inferred from indirect consideration of the properties of the photosynthetic system.

One of these classes includes the triplet, or biradical excitation, state of chlorophyll, and the related proposed chlorophyll-containing lattice in which light absorption induces the formation of conduction electrons and their corresponding holes.[11] There is no unequivocal physical evidence for either of these excited states, but they are of interest as possible means for mediating the transfer of light energy to the chemical phases of photosynthesis. Both states may be expected to be formed very rapidly in the light and to decay by slower, subsequent chemical

reactions. Both states should give rise to paramagnetic resonance absorption. Our observations are consistent with, but do not make necessary, the conclusion that the light-induced paramagnetism of chloroplasts is due to such an excited state of chlorophyll or a related physical phenomenon.

The second class of possible paramagnetic intermediates includes substances, such as DPN, which are components of the chemical electron-transport system of photosynthesis. Since such components are reversibly oxidizable in illuminated chloroplast systems, they may be expected to pass through an intermediate free-radical stage. In such a case the rate of electron transport, and the steady-state concentration of free-radical intermediate, would be a function of light intensity and would generally exhibit the behavior which we find for chloroplast paramagnetism. Our data are therefore consistent with, but fail to establish, the hypothesis that chloroplast paramagnetism is due to free radicals which are intermediates in the chemical electron-transport system associated with photosynthesis.

On the basis of present evidence we conclude that unpaired electrons associated with either or both an excited state of chlorophyll and oxidation-reduction intermediates are the cause of the paramagnetic resonance absorption which we have observed in illuminated chloroplasts. Experiments are under way which are designed to identify the substances in question and to describe their relationship to the process of photosynthesis.

We wish to thank Dr. R. E. Norberg for his helpful discussion of this work and for differential analyzer integration of certain of our data, and Mr. Alf L. Cederquist for assistance in constructing equipment.

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THE MECHANISM OF CHYMOTRYPSIN-CATALYZED REACTIONS

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INTRODUCTION

In two recent papers[1, 2] it has been suggested that the reaction of chymotrypsin with its substrates proceeds by way of three kinetically distinct steps. First there occurs a rapid initial "adsorption" of the substrate on the specificity site of the enzyme. The second step involves the acylation of a group on the enzyme by the acidic part of the substrate, with concomitant liberation of the nonacyl moiety. Finally the acylated enzyme is hydrolyzed to regenerate the free enzyme and liberate the acidic part of the substrate.

The following extension of the Michaelis-Menten scheme is both necessary and sufficient to account for all the experimental evidence concerning chymotrypsin-catalyzed reactions, except that pertaining to the effect of inhibitors other than hydrogen ions:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (\mathrm{ES}')_t, \tag{1}$$

$$(\mathrm{ES}') \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (\mathrm{ES}'')_t + \mathrm{P}', \tag{2}$$

$$(\mathrm{ES}'') \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \mathrm{E} + \mathrm{P}''. \tag{3}$$

(ES′) is the rapidly formed adsorption complex. The second complex, (ES″), is formed with liberation of the product P′, and hydrolysis of this complex leads to regeneration of the enzyme and liberation of the product P″. The evidence now available indicates that only those forms of (ES′) and (ES″) lacking a proton which ionizes off at approximately neutral pH are capable of undergoing the reactions indicated in equations (2) and (3). It is therefore necessary to consider the distribution of these complexes between their ionized and unionized forms:

$$(\mathrm{ES}') + \mathrm{H}^+ \rightleftarrows (\mathrm{ES}'\mathrm{H}^+), \qquad K_i' = [\mathrm{ES}'][\mathrm{H}^+]/[\mathrm{ES}'\mathrm{H}^+], \tag{4}$$

$$(\mathrm{ES}'') + \mathrm{H}^+ \rightleftarrows (\mathrm{ES}''\mathrm{H}^+), \qquad K_i'' = [\mathrm{ES}''][\mathrm{H}^+]/[\mathrm{ES}''\mathrm{H}^+]. \tag{5}$$

The quantities in square brackets represent the molar concentrations of the corresponding species. In equations (1) and (2), $(\mathrm{ES}')_t$ and $(\mathrm{ES}'')_t$ represent the summation of both the ionized and unionized forms of the respective complexes. As will be discussed below, the assumption of a simple ionization process in equation (4) may be an oversimplification.

It was shown in an earlier publication[2] that simple results susceptible of experimental verification are obtainable from this kinetic scheme if attention is confined to the early stages of the reaction. A slight modification of the resulting equations is necessitated by the fact that the pH dependence of the rate of reaction (2) must be included. A summary of the equations which will be useful in the discussion of the experiments reported in the present paper follows. If we represent the total

enzyme concentration by $[E]_0$ and the initial substrate concentration (assumed to remain substantially constant) by $[S]_0$, then the concentration of free enzyme, [E], is given by

$$\{(k_2' + k_3')\ [S]_0 + k_3'K_m\}\ [E] = k_3'\ K_m[E]_0 + \frac{k_2'\ K_m[S]_0[E]_0}{K_m + [S]_0} \exp\left(-\frac{(k_2' + k_3')\ [S]_0 + k_3'\ K_m}{K_m + [S]_0}\ t\right), \quad (6)$$

where $K_m = (k_{-1} + k_2')/k_1$ is the Michaelis-Menten constant, t is the time, and $k_2' = k_2K_i'/\{K_i' + [H^+]\}$ and $k_3' = k_3\ K_i''/\{K_i'' + [H^+]\}$ are apparent first-order rate constants. Measurements of the rate of appearance, $V = d\ [P']/dt$, of P' at small values of t can be employed to evaluate the rate constant

$$k \equiv \frac{(k_2' + k_3')\ [S]_0 + k_3'K_m}{K_m + [S]_0}. \quad (7)$$

If $[S]_0$ is large enough so that $(k_2' + k_3')[S]_0 \gg k_3'K_m$, then $(k_2' + k_3')$ and K_m can be evaluated in the usual way by means of Lineweaver and Burk[3] or Eadie[4] plots of the data for experiments at various substrate concentrations. The procedure employed for evaluating k from kinetic data was outlined in an earlier paper.[2]

Measurements made at times large enough so that the exponential term in equation (6) is negligibly small can be interpreted by means of the equation

$$V = \frac{d[P']}{dt} = \frac{d[P'']}{dt} = \frac{k_2'k_3'[S]_0\ [E]_0}{(k_2' + k_3')[S]_0 + k_3'K_m}. \quad (8)$$

Application of Lineweaver-Burk or Eadie plots then gives the *apparent* constants

$$K_{m\ (app.)} = \frac{k_3'}{k_2' + k_3'} K_m \quad (9)$$

and

$$\frac{1}{k_{3\ (app.)}'} = \frac{1}{k_2'} + \frac{1}{k_3'}. \quad (10)$$

It is important to note that ordinary kinetic observations, made after the decay of the exponential term in equation (6), will in general lead to apparent kinetic constants which are composites of true kinetic constants.

Niemann and his co-workers[5] in their classical work on the specificity and mode of action of chymotrypsin have used derivatives of aromatic amino acids and their structural analogues as substrates. We have extended some of their observations on the hydrolysis of esters and amides of tyrosine derivatives and have also studied the chymotrypsin-catalyzed hydrolysis of nitrophenyl esters. This latter reaction was discovered by Hartley and Kilby,[6] and some of its applications to the study of the mode of action of chymotrypsin have been described by Balls and his co-workers.[7]

The experimental information recorded in this paper was obtained by a novel approach to the problem of enzyme kinetics, involving the use of spectrophotometric fast-reaction techniques to study the kinetics of the establishment of the steady state. Gutfreund[8] has shown recently how the elegant stopped-flow tech-

niques of Chance and Gibson can be employed to follow the steps involved in the formation and decomposition of enzyme-substrate complexes in hydrolytic systems. Although the fast-reaction techniques in their present state of development do not permit the attainment of the experimental accuracy realizable in kinetic studies on slower processes, nevertheless it has been found to be possible to obtain data which add materially to our understanding of the mechanism of chymotrypsin action.

EXPERIMENTAL METHODS AND RESULTS

All experiments were carried out at 25°, in solvents containing 20 per cent (V/V) isopropyl alcohol and 0.1 M NaCl. pH measurements were made with a glass electrode, using a pH 7.0 aqueous phosphate buffer as standard. Two enzyme preparations, several times recrystallized, were employed. (The enzyme preparations were kindly supplied by B. S. Hartley and J. I. Harris, of the University of Cambridge.)

The Initial Adsorption Step.—Gutfreund[8] has shown how the method of "initial acceleration" can be employed to estimate k_1 of equation (1). This method was applied to the reaction of chymotrypsin with benzoyl-L-tyrosine ethyl ester. (The authors are indebted to J. S. Fruton, of Yale University, for a sample of this substance.) It was found that the steady-state concentration of (ES′) was formed in something less than the smallest time (0.002 second) observable in the stopped-flow apparatus. k_1 is related to the intercept, τ, of the steady-state plot with the time axis by the equation

$$\tau = \frac{1}{k_1\{[S]_0 + K_m\}}. \tag{11}$$

In the present experiments, $[S]_0 = 1.2 \times 10^{-4}\ M$. Taking the value[9] of K_m as $1.2 \times 10^{-4}\ M$, with $\tau \leq 2 \times 10^{-3}$, we then find that $k_1 \geq 2 \times 10^6$ liters mole^{-1} sec^{-1}.

Experiments with 2,4-Dinitrophenyl Acetate.—Our previously reported[2] measurements with p-nitrophenyl acetate have been extended by kinetic observations of the hydrolysis of 2,4-dinitrophenyl acetate. The low pK of dinitrophenol makes it possible to perform experiments at lower values of pH than can be employed with p-nitrophenyl acetate. The experimental procedure was exactly as previously described. All solutions were buffered by 0.05 M phosphate.

The low solubility of the substrate prevented the use of values of $[S]_0$ higher than $1.5 \times 10^{-3}\ M$, which is approximately the value of K_m for this system. This, together with the difficulties of the fast-reaction technique, has made it impossible to secure values of K_m and k_2' which are as reliable as is desirable in enzyme work; the accuracy obtained is, however, adequate for our present purposes.

Experiments were carried out in the pH range 6.1–7.2. In all cases the formation of the steady-state concentration of (ES″) was found to follow first-order kinetics, as would be expected from the form of equation (6). Several runs were made at each value of $[S]_0$ and pH, and the rate constants were averaged. Eadie plots for three values of pH are given in Figure 1. Since in this system $k_2' \gg k_3'$, these plots lead directly to values for k_2' and K_m. The experimental data are summarized in Table 1; the individual rate constants deviate on the average ±3 per cent from the lines defined by the values of k_2' and K_m listed in the table. The

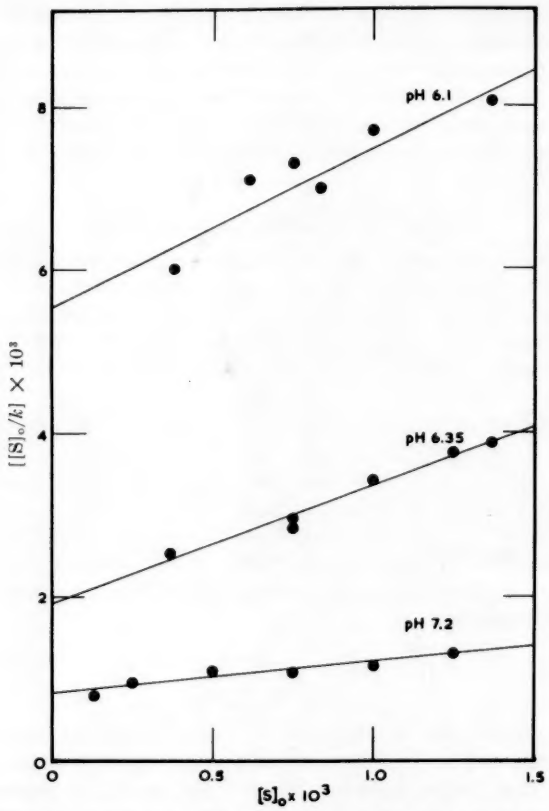

FIG. 1.—Eadie (*J. Biol. Chem.*, **146**, 85, 1942) plots for three values of pH for the reaction of chymotrypsin with 2,4-dinitrophenyl acetate. $[S]_0$ is the molar initial substrate concentration; k is defined by eq. (7).

variation of k_2' with hydrogen-ion concentration is indicated by the plot in Figure 2, the data for these experiments being indicated by open circles.

TABLE 1

KINETICS OF FORMATION OF (ES′) IN THE CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF 2,4-DINITROPHENYL ACETATE AT 25°

pH	k_2' (Sec^{-1})	K_m (Moles/Liter, $\times 10^3$)	Range of $[S]_0$ (Moles/Liter, $\times 10^3$)	Standard Deviation (Per Cent)
6.10	0.52	2.90	0.37–1.37	±3.4
6.15	0.60	2.03	.25–1.87	±3.5
6.35	0.67	1.33	.37–1.37	±2.5
6.70	2.42	5.50	.37–1.25	±1.6
7.20	2.61	2.16	0.13–1.25	±5.3

Experiments in p-Nitrophenol Buffer.—Two sets of measurements of the second stage in the hydrolysis of *p*-nitrophenyl acetate were made, using 10^{-3} *M* *p*-nitro-

phenol as buffer at pH 6.6. In these experiments the primary aim was to determine the amount of hydrogen ions taken up or liberated during the reaction, so that it was necessary to calibrate the optical system by means of standard solutions of *p*-nitrophenylate ion. An individual run from one set of measurements is reproduced in Figure 3. The solid line is calculated as the superposition of an exponential and a linear term, the latter being due to the final steady-state liberation of hydrogen ions plus electrical drift, and the circles are observed data. A sufficiently accurate estimate of the total color change is obtained by extrapolating the linear portion of the record back to the time of starting the reaction.

The results of these experiments are given in Table 2. The two sets of measurements agree well in indicating a loss by the buffer of very nearly one-half mole of protons per mole of enzyme present.

Since the enzyme was present in relatively high concentration, a blank experiment was performed to show that the enzyme itself exerted very little buffering action in the absence of the substrate. One-hundredth of a milliliter of a 10^{-2} *M* solution of *p*-nitrophenol in isopropyl alcohol was added to 2.5 ml. of a solution

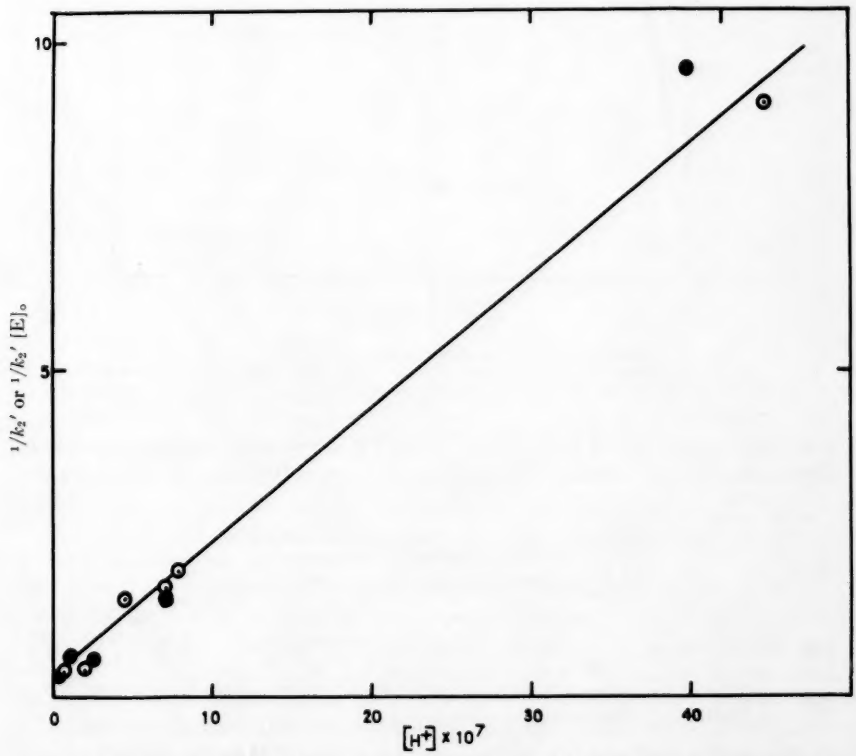

FIG. 2.—Plot of $1/k_2'$ for the reaction of chymotrypsin with 2,4-dinitrophenyl acetate (○), and of $1/k_2'$ $[E]_0$ (in arbitrary units) for the reaction of chymotrypsin with acetyl-L-tyrosine amide (●), against hydrogen-ion concentration $[H^+]$.

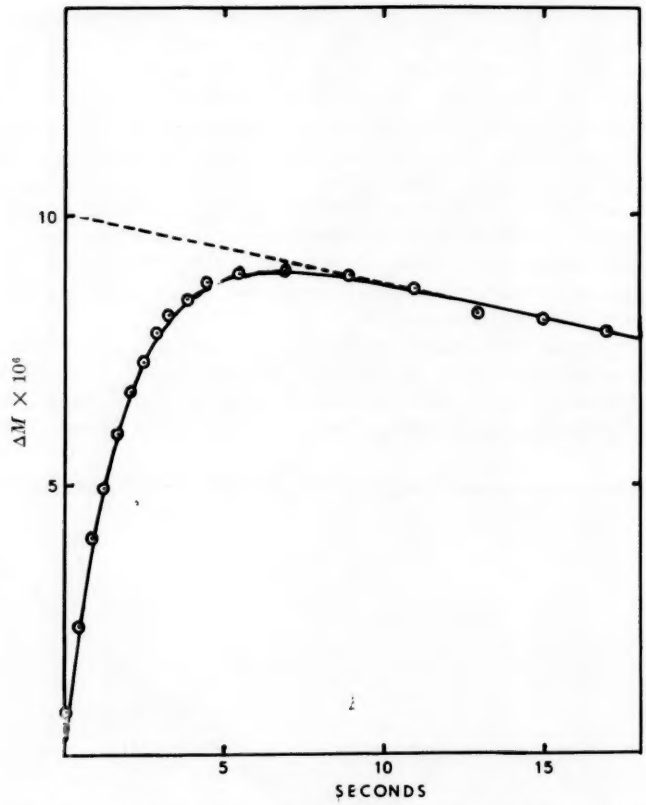

FIG. 3.—Record of change in concentration of *p*-nitrophenylate ion, ΔM, during a reaction of chymotrypsin (2×10^{-5} *M*) with *p*-nitrophenyl acetate (2.5×10^{-3} *M*) in *p*-nitrophenol buffer (1×10^{-3} *M*).

containing *p*-nitrophenol and enzyme at pH 6.6 in the same concentrations as in the experiments of Table 2. The observed increase of 0.021 in the optical density

TABLE 2

CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *p*-NITROPHENYL ACETATE IN 0.001 *M* *p*-NITROPHENOL BUFFER, pH 6.6, 25° ($[S]_0 = 0.0025$ *M*)

Number of Runs	$[E]_0$ (Moles/Liter, $\times 10^3$)	$\Delta[H^+]/[E]_0$	k_2' (Sec^{-1})
3	2.0*	0.49 ± 0.04	0.57 ± 0.02
9	3.5†	0.49 ± 0.03	0.63 ± 0.03

* Estimated from refractive increment, taking specific refractive increment = 0.00185, MW = 23,500.
† Salt-free lyophilized enzyme weighed out directly.

of the solution corresponds to an increase of 1.1×10^{-6} *M* in the concentration of *p*-nitrophenylate ion, whereas the added *p*-nitrophenol amounted to 4×10^{-5} *M*. Since only a negligible fraction of this increase in color is due to imperfect buffering by the *p*-nitrophenol originally present, this blank experiment shows that approxi-

mately one-fortieth of the color change observed in the experiments in Table 2 was due to buffering by the enzyme *in its original state.*

Experiments with Acetyl-L-Tyrosinamide.—In order to make a comparison of the pH dependence of chymotrypsin-catalyzed hydrolysis of esters and amides, it was necessary to extend the measurements of Thomas, MacAllister, and Niemann[10] on the steady-state kinetics of the hydrolysis of acetyl-L-tyrosinamide to the acid side of pH 6.6. In these experiments the composition of the reaction mixtures was the same as that used in the experiments with dinitrophenyl acetate, except that 0.025 M maleate buffers were employed. (The acetyltyrosinamide used in much of this work was kindly supplied by Miss L. K. Mee.) Initial substrate concentrations in the range $2\text{–}23 \times 10^{-3}$ M were employed, the upper limit being imposed by the solubility of the substrate. Since the apparent Michaelis-Menten constant for this system in the solvent we employed is approximately 10^{-1} M, it is evident that the situation is extremely unfavorable for obtaining accurate values of $K_{m(\text{app.})}$ and $k_3'_{(\text{app.})}[\text{E}]_0$ (see eqs. [8]–[10]).

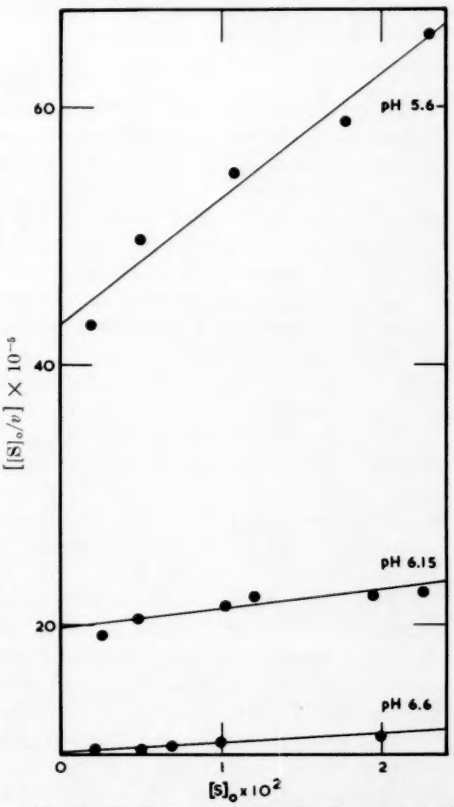

FIG. 4.—Eadie (*op. cit.*) plots for three values of pH for the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine amide. $[S]_0$ is the molar initial substrate concentration.

The reactions were followed by estimating the ammonia formed by the ninhydrin method,[11] the colors being determined with a Unicam SP.500 spectrophotometer. The high sensitivity of the ninhydrin method makes it possible to restrict observations to the early part of the reaction, where no more than 5 per cent of the substrate has been hydrolyzed. The rates given are therefore strictly initial rates.

Measurements were made at various values of the pH in the range 5.6–7.5; the results at three values of the pH are given in the form of Eadie plots in Figure 4. As shown in the discussion below, it is very probable that for this system $k_3' \gg k_2'$, so that the constants obtained from these Eadie plots are actually $k_2'[\text{E}]_0$ and K_m. The values of these constants are summarized in Table 3 and define lines which reproduce the original data with the standard deviations given in the last column of the table. The variation of $k_2'[\text{E}]_0$ with pH is indicated by the filled circles in Figure 2.

TABLE 3

CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT 25°

pH	$k_2'[E]_0$ (Sec^{-1}, $\times 10^2$)	K_m (Moles/Liter)	Range of $[S]_0$ (Moles/Liter, $\times 10^3$)	Standard Deviation (Per Cent)
5.6	0.104	0.045	0.2–2.3	±2.6
6.15	0.67	.133	.3–2.3	±2.8
6.6	1.79	.182	.2–2.0	±1.3
7.0	1.61	.105	.1–2.7	±1.6
7.5	3.38	0.181	0.3–2.6	±1.5

DISCUSSION

At a recent conference[12] on enzyme mechanisms a number of authors summarized evidence that both the hydroxyl group of a serine residue and the imidazole group of a histidine residue are at least part of the catalytic site of the hydrolytic enzymes trypsin, chymotrypsin, and cholinesterase. The work described in this paper is part of a program designed to identify the nature and sequence of the chemical reactions which occur between the substrate and the catalytic groups on the enzyme. Through our studies of the reactions of chymotrypsin with *p*-nitrophenyl acetate and 2,4-dinitrophenyl acetate, we have developed a method which allows us to investigate separately the kinetics of the second and third steps of the enzyme reaction described in equations (2) and (3). Furthermore, we have made an attempt to determine which step is rate-controlling for reactions of chymotrypsin with two of its specific substrates, acetyl-L-tyrosine ethyl ester and amide.

Two important conclusions about the mechanism of chymotrypsin reactions, in addition to those discussed in our previous paper, can be drawn from the experimental results presented here. In the first place, the acylation reaction, in addition to the hydrolysis of the acylated enzyme, is inhibited by the protonation of a basic group on the catalytic site; in the second place, the pK of this basic group is changed to a higher value as a result of the acylation of the enzyme.

The form of the pH dependence of k_2' shown in Figure 2 indicates a value of approximately 6.7 for pK_i', where K_i' is the inhibition constant defined in equation (4). It is, however, not clear whether the change of rate with pH can be represented by a simple titration curve or whether there is a more sudden drop in rate between pH 6.7 and 6.4 than can be accounted for by a simple titration curve. In this connection, it may be noted that our previous work with *p*-nitrophenyl acetate indicated only a small change in k_2' between pH 7.7 and 6.5. A considerable volume of experimental data will be required to settle this point, and at present it must be accepted that the assumption of a single classical ionization in equation (4) is not strongly supported by experiment. We have previously shown that the third step of the reaction sequence—the hydrolysis of the acyl enzyme—is controlled by a group with $pK_i'' = 7.3$ (cf. eq. [5]).

Additional insight into the mechanism of the catalysis was obtained from experiments designed to demonstrate whether hydrogen ions are liberated or taken up during the reaction of the enzyme with *p*-nitrophenyl acetate. These experiments are summarized in Table 2.

If the acetylation of the enzyme takes place at an —SH or an —OH group, the reaction controlled by the rate constant k_2' is

$$\begin{matrix} E—SH \\ E—OH \end{matrix} + O_2N\cdot C_6H_4O\cdot CO\cdot CH_3 \rightarrow \begin{matrix} E—S\cdot CO\cdot CH_3 \\ E—O\cdot CO\cdot CH_3 \end{matrix} + O_2N\cdot C_6H_4\cdot OH. \quad (12)$$

In general, the pH will be such that part of the colorless *p*-nitrophenol formed will undergo ionization, but if the solution is well buffered by *p*-nitrophenol, all the protons so formed will be taken up by *p*-nitrophenylate ions and there will be no increase in the concentration of the colored species.[13] On the other hand, if the acetylation involves a protonated (or partially protonated) group, the reaction is

$$E = \dot{N}H_2 + O_2N \cdot C_6H_4O \cdot CO \cdot CH_3 \rightarrow E = N \cdot CO \cdot CH_3 + H^+ + O_2N \cdot C_6H_4 \cdot OH, \quad (13)$$

and there should be a decrease in the concentration of the colored *p*-nitrophenylate ion, the extent of the decrease depending on the extent of protonation of the group which becomes acetylated.

The data for the two experiments summarized in Table 2 show that the concentration of *p*-nitrophenylate ion actually *increased*, indicating a net loss of protons from the buffer to the extent of approximately one-half mole of proton per mole of enzyme. This result makes it appear most unlikely that a basic group is acetylated, since at pH 6.6 any basic group in the enzyme would be at least partly protonated.

The conclusion cannot be avoided that the protons removed from the buffer are taken up by the enzyme. In view of the fact that the enzyme exerts only a negligible buffering action in its initial state, it is necessary to assume that the pK of some group on the enzyme is increased by the acetylation step. In the case of dinitrophenyl acetate, the rate of the acetylation step is controlled by the extent of protonation of a group having a pK of approximately 6.7, while the subsequent hydrolysis of the acetylated enzyme is controlled by a group having a pK of 7.3. These facts strongly suggest that these two groups are one and the same, and that it is this group which takes up protons following the acetylation. It can be readily shown that if a group with a final pK of 7.3 takes up one-half mole of protons per mole at pH 6.6, as a result of a change in pK, its initial pK is approximately 6.4. A value of pK_i' of this magnitude may not be inconsistent with our earlier work on *p*-nitrophenyl acetate.

The results of the measurements of the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinamide over the pH range 5.6–7.5 are plotted as filled circles in Figure 2. It can be seen clearly that the pH dependence of the rate of amide hydrolysis is markedly similar to that of the acylation step in the reaction with dinitrophenyl acetate, even to the indication of anomalous behavior between pH 6.6 and 6.4. This similarity gives support to the suggestion that the formation of (ES″) is the rate-determining step in the amide hydrolysis. The fact that the over-all rate of the chymotrypsin-catalyzed hydrolysis of acetyltyrosine ethyl ester is approximately 1,000 times larger than that of the corresponding amide is also readily explained on the basis of this suggestion.

Hammond and Gutfreund[1] have found $pK_i = 6.85$ for the over-all ester hydrolysis. It appears that the considerably higher rate of the acylation step with the ester substrate brings about a situation in which k_2 and k_3 are of the same order of magnitude, with a consequent contribution of both steps to the control of the over-all rate.

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[12] See *Discussions Faraday Soc.*, vol. **20**, 1955.

[13] The interpretation of an experiment of this type given in our previous publication (*op. cit.*) is in error. The results of that experiment are in qualitative agreement with those of similar experiments reported in the present paper.

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*STUDIES ON SIALIC ACID OF SUBMAXILLARY MUCOID**

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In recent years an increasing body of information has accumulated on compounds of great biological importance, named by some "sialic acid," by others "neuraminic acid," "gynaminic acid," "hemataminic acid," "lactaminic acid," or "serolactaminic acid."[1] These substances, believed to be identical and referred to here as "sialic acid," are encountered as an important constituent of many mucoids, of complex lipids, and of certain oligosaccharides. Sialic acid has been reported in serum, urine, saliva, meconium, brain, milk, colostrum, mammary gland, etc. Increases in serum sialic acid have been established in the presence of inflammatory diseases and cancer. The acid plays a key role in influenza virus hemagglutination. Although the structure of sialic acid has been the object of study in many laboratories, twenty years of activity on the problem have not brought a satisfactory answer. Chief obstacles to success were the difficulty in preparing the naturally occurring material in large quantity and the inability to degrade it to identifiable products. The acid can be isolated after weak acid hydrolysis as well as enzymatic hydrolysis of the proper starting materials. Some investigators[2] have obtained the deacetylated methyl glycoside by metanolysis and hydrolysis. However, enzymatic degradation is preferable for obtaining the compound in good yield and as it probably occurs in nature. Work in this laboratory was undertaken following the isolation of an apparently homogeneous mucoid from submaxillary gland[3] and the hydrolysis of it by an extract of pneumococci.[4] The present communication deals with attempts to establish the mecha-

nism of enzymatic cleavage of BSM by RDE. During this study, an enzymatic degradation of sialic acid also was observed, which sheds new light on the structure of sialic acid.

EXPERIMENTAL

Isolation of BSM.—BSM was prepared by extraction at 0° of acetone powders of bovine submaxillary gland with aqeuous 6 *N* urea, adjusted to pH 9. The clear extracts obtained after centrifugation were acidified to pH 3.5, at which point a mucin clot appeared. The latter was purified by dissolving in equal amounts of *N* calcium acetate and 0.5 *N* acetic acid, and stirring with a mixture of chloroform and amyl alcohol (3:1, v:v) until fully deproteinized. The aqueous phase was fractionated by addition of ethanol. Mucoids precipitated between 46 and 78 per cent ethanol contained the same proportion of sialic acid. The homogeneous mucoid precipitated at 54 per cent ethanol was eventually selected for further study, as it accounted for 60 per cent of the total yield (9 gm. of mucoid from 100 gm. of acetone powder).

Enzymes.—RDE was prepared from filtrates of *Vibrio cholerae* in the laboratories of Sharp and Dohme, Philadelphia, Pennsylvania, and from autolysates of pneumococcus, type II, according to methods described elsewhere.[5]

Enzymatic Hydrolysis of BSM.—An aqueous solution of 600 mg. of BSM was incubated with 30 mg. of enzyme (either source), kept 16–48 hours under toluene at 37° without addition of buffer solution. The digest was dialyzed against frequently changed distilled water. The dialyzable and nondialyzable portions were isolated and analyzed after concentration and lyophilization.

Isolation of Sialic Acid.—The dialysate was shaken with Dowex 50 (H+) and chromatographed on Dowex 1–10 (formate). Sialic acid was eluted with 0.07 *N* formic acid in a single peak. After evaporation under reduced pressure, followed by lyophilization, the eluate was dissolved in absolute methanol and slowly brought to turbidity by addition of ether and petroleum ether. Crystals appeared on standing near 0° after a few days. In other experiments, slow evaporation of the solvent in a desiccator at room temperature resulted in the appearance of crystalline deposits on the walls of the vessel, which were then harvested and repeatedly recrystallized.

Analytical Methods.—Sialic acid was evaluated by Bial's orcinol, Ehrlich's dimethylaminobenzaldehyde, and Tracy's manometric technique.[6] Hexosamine was determined by the methods of Elson and Morgan,[7] as well as of Gardell;[8] reducing values by hypoiodite[9] and ferricyanide[10]; and nitrogen by the Markham modification of the micro-Kjeldahl procedure. Elementary analyses, acetyl determinations (*p*-toluenesulfonic acid), and methoxyl analyses were carried out by Schwarzkopf Microanalytical Laboratories, Woodside, New York.

RESULTS AND DISCUSSION

Inhibition of Influenza Virus by BSM.[11]—One to two micrograms of BSM inhibited 5 hemagglutinating units of indicator virus (PR8, heated 56° for 30 minutes); 2–5 μg. of BSM inhibited 5 hemagglutinating units of indicator virus LEE and active NWS virus. The inhibitory capacity of BSM was abolished by prior treatment with active influenza virus, by RDE, and by crystallized trypsin (Tryp-

tar, Armour) but was unaffected by culture filtrates of *Clostridium tertium*. Urinary mucoprotein (Tamm and Horsfall) was used as a standard of comparison in assays of BSM, 1–2 μg. inhibiting 5 hemagglutinating units of the same indicator virus.

Characteristics of BSM.—Table 1, Column I, lists chemical analyses. BSM was completely water-soluble. A 0.35 per cent solution had a relative viscosity of 3.65 at 37°. Its specific rotation was −4° at 27°. The ultraviolet spectrum showed no maxima. BSM was homogeneous and monodisperse as tested by electrophoresis (pH 4.5 and 8.6) and by ultracentrifugation[12] (pH 7). Tests for sulfate and phosphate were negative. Maximum reducing values after acid hydrolysis (*N* HCl, 100°, 4 hours) were equivalent to 16 per cent glucose. Hexosamines were identified as galactosamine and glucosamine (ratio 6:1, 15.4 per cent by the method of Gardell). Dische's carbazole test for uronic acid was negative. Tests for hexose (anthrone) and fucose gave insignificant values (1.3 and 0.5 per cent,

TABLE 1

ANALYSES OF BSM AND OF RDE-TREATED BSM FRACTIONS*

	BSM (I) (Mg.)	Nondialysable (II) (Mg.)	Dialysable (III) (Mg.)	Recovery (II) + (III) (Per Cent)
Weight	595.5	486.7	114.1	101%
Sialic acid:				
Ehrlich	148 (24.8%)	73.5 (15%)	36.2 (31.7%)	74%
Bial	160 (27.8%)	77.3 (15.9%)	36.3 (31.8%)	71%
Tracy	188 (31.6%)	92.5 (19%)	69.7 (61.2%)	86%
Total hexosamine (Elson-Morgan)	109.8 (18.6%)	94.4 (19.4%)	9.1 (8%)	95%
Hypoiodite (glucose) without hydrolysis)	23.8 (4%)	13.6 (2.8%)	34.3 (30%)	201%
$Fe(CN)_6$ (glucose) (without hydrolysis)	23.8 (4%)	43.6 (8.9%)	5.9 (5.2%)	206%
Nitrogen	54.2 (9.1%)	44.6 (9.2%)	3.7 (3.2%)	90%
Inorganic (Ca^{++})	....	....	9.5 (8.3%)	..

* 595.5 mg. BSM incubated with 30 mg. RDE, 48 hours, 37° C. (Fraction II corrected for contribution of 30 mg. RDE).

respectively). The acetyl content of BSM was 9.80 per cent. (*Theory:* 6.87 per cent if sialic acid is monoacetylated, 9.66 per cent if sialic acid contains an additional *O*-acetyl group. Theoretical values were calculated on the basis of 17.0 per cent hexosamine [av.] and 28.1 per cent sialic acid [av.].) This result indicates that BSM contains diacetylated (bovine) sialic acid.[13] When BSM was left standing at room temperature for 1 hour in the presence of 0.02 *N* Na(OH), the alkalilabile *O*-acetyl group appeared to have been hydrolyzed, as evidenced by an acetyl value of 7.53 per cent of reisolated BSM with unchanged sialic acid content (27 per cent). The amino acids after hydrolysis with 6 *N* HCl for 16 hours and determination by two-dimensional paper chromatography were valine, proline, alanine, threonine, serine, glycine, glutamic acid, aspartic acid, and lysine.

Action of RDE on BSM.—Digestion of BSM with RDE was followed by increases in hypoiodite consumption. The pH optimum of the reaction was between pH 4.5 and 6.0. No hydrolysis occurred below pH 4.0. In 3 hours, 53 per cent of BSM sialic acid was hydrolyzed in 0.003 *M* phosphate buffer, pH 6, 37° C. Digestion was accompanied by a sharp decrease in relative viscosity of BSM from 3.65 to 2.30 in 10 minutes, sloping off to 1.94 in 120 minutes. The high viscosity retained indicates that even after loss of approximately 50 per cent sialic acid, the

molecule retained considerable asymmetry. No significant changes in pH or increase in free amino or free hexosaminidic end groups[14] occurred.

Analysis of the nondialyzable mucoid core (see Table 1) indicated that this portion was in the main responsible for the increased ferricyanide reducing values observed during incubation. On the other hand, the core showed no comparable increase in hypoiodite consumption. Reduction of the core by sodium borohydride, however, caused no appreciable change in either reducing value. Ferricyanide reducing values varied with the amount of sialic acid released, being nearly 18 per cent (as glucose) when all the sialic acid had been removed from BSM by acid hydrolysis (0.5 *N* H_2SO_4, 2 hours, 100°). The hypoiodite reducing value of BSM freed of sialic acid was not greater than of unaltered BSM. Sodium borohydride still was ineffective in causing a change in the reducing values. These data indicate the failure of either enzyme or acid to lay open reducing end groups within the mucoid core concomitant with the hydrolysis of sialic acid. The nature of the group responsible for the ferricyanide reduction is not known.

The dialyzable portion, on the other hand, was responsible for the increase in hypoiodite consumption recorded during enzymatic incubation. The liberation of sialic acid was responsible for hypoiodite reduction. The fact that color reactions used for assay of sialic acid in the dialysate gave low recoveries, whereas Tracy's method did not, eventually led to the detection of another enzyme contained in RDE, which is responsible for a conversion of sialic acid to N-acetylglucosamine and another yet unknown compound. This will be discussed later. The ratio of sialic acid (Tracy) to hypoiodite consumption was nearly unity. Therefore, the major event during enzymatic hydrolysis of BSM attendant to the cleavage of sialic acid is the liberation of a glycosidic bond. Further evidence for this came from experiments with sodium borohydride. The latter caused no change in colorimetrically measured sialic acid in either BSM or the mucoid core, whereas the dialysate treated similarly lost all its Bial's and Ehrlich's color. This indicates that the reducing end of sialic acid must be linked to the mucoid. Furthermore, since there was no change in the free amino group content of BSM following enzymatic hydrolysis, the bond split must be O-glycosidic rather than N-glycosidic, as suggested by Gottschalk.[15] The name "sialidase" is proposed for the enzyme responsible for this action. On the other hand, since the destruction of receptor sites on mucoids capable of inhibiting influenza virus hemagglutination can be mediated by enzymes other than sialidase, it is proposed that the general term "RDE" be retained to describe those cases where destruction of biological activity alone is determined.

Characterization of Isolated Sialic Acid.—Sialic acid isolated as the methyl ester from the dialysate gave the following analysis:

	C	H	N	OCH_3	COCH
Calculated for $C_{13}H_{23}NO_{10}$	44.14	6.56	3.96	8.78	12.49
Found	44.94	6.66	4.08	8.41	12.20

Melting point: 169°–171° uncorr. $[\alpha]_D^{24} = -32°$ (water)
Yield: 40 per cent of colorimetrically estimated sialic acid, or a total of 10 per cent of BSM

The rotation as well as the infrared spectrum of this compound were nearly identical with the ones reported for sialic acid free of methoxyl groups.[16] The crystalline

material is believed to be a methyl ester. The ester gave a strong ferric hydroxamate test, in contrast to sialic acid. On paper chromatography in butanol, acetic acid, and water (50:12:25), the ester exhibited two spots, R_f 0.48 and 0.58 (sialic acid, R_f 0.24). Esterification occurred in crystallization, as the spot of R_f 0.24 was the only one found in paper chromatograms of the dialysate. The ester gave a brown spot with the benzidine trichloroacetic acid spray reagent,[17] whereas the color of the acid spot was blue. Hypoiodite titration equivalent to 1 mole of aldehyde, as well as the failure of the compound to exhibit an ultraviolet spectrum, exclude the proposed 2 keto-, 3 deoxy-, 5 deoxy-, 5 amino-, 5-acetaminononononic acid[15] as a possible structure of sialic acid.

Enzymatic Degradation of Sialic Acid.—Table 2 enumerates the changes occurring upon incubation of sialic acid with RDE. The loss of Bial's orcinol as well as the increase in hexosamine were found to be a function of time and enzyme concentration. The stoichiometric relationship between the loss of sialic acid (0.084 μM) and the appearance of hexosamine (0.089 μM), accompanied by an increase in reducing value (0.152 μM), is interpreted as an indication of internal glycosidic splitting. One product of the cleavage was N-acetylglucosamine, while the other has yet to be identified. Evidence for the former was obtained by paper chromatography as well as by absorption-spectrum analysis. Using Partridge's hexos-

TABLE 2

NET CHANGES IN ANALYSIS OF SIALIC ACID FOLLOWING INCUBATION WITH RDE* (μM)

	Initial	48 Hours	Change
Bial's orcinol (sialic acid)	0.260	0.176	−0.084
Hexosamine	.013	.102	+0.089
Hypoiodite	0.244	0.396	+0.152

* 1 mg. of methyl sialate incubated with 5 mg. of RDE (*Vibrio cholerae*), 48 hours, 37° C., under toluene, 0.003 *M* phosphate buffer, pH 5. Values expressed in micromoles after subtraction of enzyme blanks.

amine spray reagent,[18] authentic N-acetylglucosamine as well as the material obtained from the enzymatic degradation had identical R_f values. Acid hydrolysis converted this compound into glucosamine, having an R_f value indistinguishable from authentic D-glucosamine HCl when tested in two solvent systems. After ninhydrin oxidation, both compounds, when tested by the procedure of Stoffyn and Jeanloz,[19] gave arabinose. Sialic acid therefore is believed to contain a glucosamine moiety. Additionally, absorption spectra of the Elson-Morgan hexosamine chromogens were compared. Sialic acid does not show the absorption spectrum of the chromogen of N-acetylglucosamine, and acid-hydrolyzed sialic acid similarly does not resemble glucosamine. Upon incubation with enzyme, however, spectra typical for the chromogen of N-acetylglucosamine and glucosamine (after acid hydrolysis) appeared. Appropriate enzyme blanks did not exhibit the Elson-Morgan chromogen. The spectra were, furthermore, re-examined 48 hours after initial color development, to rule out anomalous hexosamine chromogens obtained by interaction of aldehyde and amine groups other than hexosamine.[20] The increase in reducing value following the degradation of sialic acid is interpreted as due to a cleavage of a glycosidic bond, linking N-acetylglucosamine with a yet to be characterized aldehyde. This aldehyde should contain an α-hydroxycarboxylic acid moiety, accounting for the low pK of sialic acid as well as for the stoichio-

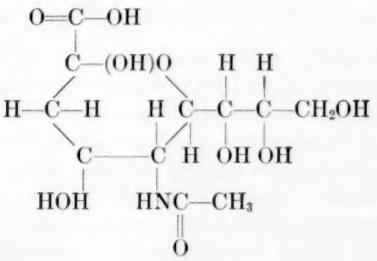
metric liberation of CO_2 with acid hydrolysis. This fragment is $C_4H_6O_4$, if sialic acid is $C_{12}H_{21}NO_{10}$. Only two structures can be written for this, namely, malic acid semialdehyde or α-hydroxy, α-methylmalonic acid semialdehyde. Since sialic acid contains a free hexosaminidic end group,[16] the glycosidic bond could be only on carbons 3–6 of the hexosamine moiety. Any acceptable structural proposal for sialic acid, however, must accommodate the fact that all efforts to obtain hexosamine by acid hydrolysis have remained unsuccessful, apparently because humin formation precedes glycolysis.

SUMMARY

A homogeneous mucoid (BSM) of bovine submaxillary gland has been described. RDE, by a mechanism of O-glycosidic cleavage, splits BSM to yield sialic acid. Sialic acid is a glycoside, yielding, upon incubation with filtrates of *V. cholerae*, N-acetylglucosamine and a yet uncharacterized reducing compound.

Addendum.—After completion of this manuscript, the following additional information has been obtained by us:

Sialic acid is split by extracts of *V. cholerae* to yield equimolar amounts of N-acetylglucosamine and pyruvic acid. The presence of pyruvic acid has been established by following the enzymatic incubation by the 2,4-dinitrophenylhydrazine reaction for pyruvic acid. Sialic acid does not interfere with this determination. The absorption spectra of the 2,4-dinitrophenylhydrazones of pyruvic acid and of the material liberated by the enzyme were identical. Evidence points to the splitting of sialic acid by an aldolase mechanism, indicating that the compound has a structure similar to the one proposed by Gottschalk:[15]



Similarly to α-keto acids, the compound reacts with semicarbazide at room temperature, although the rate of reaction is substantially slower than with other α-keto acids. This indicates a pyranose ring structure, which is also suggested by the result of periodate studies.[16] The hypoiodite reduction of sialic acid in the absence of an aldehyde group is anomalous. Sialic acid is attached glycosidically to the mucoid core, not through its aldehydic reducing end but through its carbonyl group located at C-2.

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† Taken in part from a thesis to be submitted by Ralph Heimer in partial fulfillment of the requirements for the Ph.D. degree, Faculty of Pure Science, Columbia University, New York. Abbreviations used: BSM = bovine submaxillary mucoid; RDE = receptor destroying enzyme.

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*AN IDENTICAL DUPLEX STRUCTURE FOR POLYNUCLEOTIDES**

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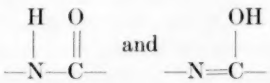
A structure for the sodium salt of deoxyribonucleic acid (DNA) conceived by Watson and Crick[1] consists of two polynucleotide chains, which are running in opposite directions and are wound about a common helical axis. In order to satisfy the geometrical requirements of this structure, the occurrence of the bases adenine, guanine, thymine, or cytosine on one chain requires the respective occurrence of thymine, cytosine, adenine, or guanine on the other, so that the two chains bear a complementary relationship to each other. The planes of the bases lie perpendicular to the axis of this double helix, 3.4 A apart. This structure is in agreement with the analytical and X-ray data for DNA. It is possible to construct helical two-chain models for polynucleotides[2] other than the above complementary duplex. These models share with the Watson-Crick structure the features of two sugar-phosphate chains winding about a common helical axis and hydrogen-bonded base pairs lying perpendicular to that axis. The models differ from the Watson-Crick formulation in the manner in which the purine and pyrimidine bases of the two opposite chains are hydrogen-bonded to each other.

We wish to describe in this communication a further example of a hypothetical double helix: a duplex built up of two polynucleotide chains of *identical*, rather than complementary, base sequence. Along one chain adenine, guanine, cytosine, and thymine (uracil) may follow one another in any arbitrary order. The base pairing is shown in Figure 1, where it is seen that each base on one chain forms two hydrogen bonds with an identical base on the second chain and that the

N_{base}—C_{sugar} bonds of each pair lie in the same position relative to each other. The adenine-adenine, cytosine-cytosine, and thymine (uracil)-thymine (uracil) pairings are those previously described[2] as Nos. 1, 18, and 23; the guanine-guanine pair requires the enol form of this base for optimum hydrogen bonding, i.e., the tautomer different from that assumed to exist in the Watson-Crick structure. The difference in free energy between the two structures



is difficult to estimate, especially in ring systems such as those with which we are concerned here, but it is not unlikely that a guanine molecule will assume that tautomeric form which will permit it to form the best hydrogen bonds in a given environment.

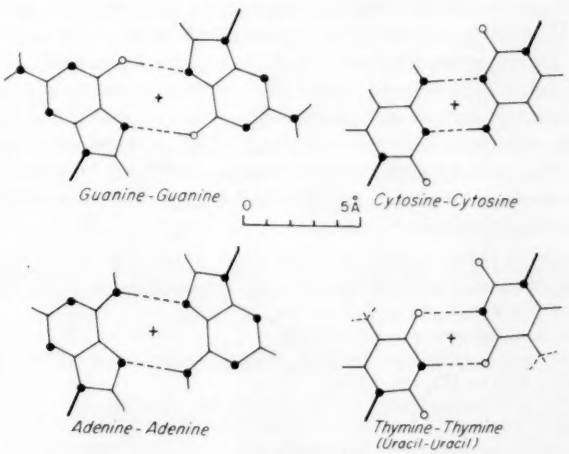


FIG. 1.—The base pairing on the identical duplex. The nitrogen atoms are represented as filled circles and the oxygen atoms as open circles. The hydrogen bonds are the dashed lines, and the bond from base nitrogen to the sugar is shown as the heavy line.

We have investigated polynucleotide structures based on the pairings shown in Figure 1 with molecular models and have found that if successive base pairs are separated by 3.4 A, it is possible to connect these successive pairs with either ribose or deoxyribose phosphate esters and construct a satisfactory right-handed double helix. Successive N_{base}—C_{sugar} bonds are related to each other by a rotation of about 45°. If this rotation is exactly 45°, the repeat distance along the axis is therefore 13.6 A if the two polynucleotide chains run in the same direction or 27.2 A if they run in opposite directions. There are thus three different possible structures based on the pairings of Figure 1. The diameter of a chain (taken as twice the largest radial atom co-ordinate plus its van der Waals diameter) is about 23 A, or 2 A smaller than that of the Watson-Crick DNA structure.

Although the identical duplex cannot be the structure of those types of DNA on which X-ray diffraction studies have been carried out[3] (e.g., sperm, bacteriophage, thymus), it is not excluded that this helix resembles the configuration of DNA or ribonucleic acid (RNA) at some stage of the biological reduplication process. It is interesting in this connection that Rich and Watson[4] concluded, from an X-ray diffraction study of rather poorly oriented RNA fibers, that (1) the bases are stacked roughly perpendicular to the fiber axis; (2) the repeat distance along the fiber axis is 25–28 A; (3) the structure is helical; (4) the diameter of the fiber is 21–25 A. Calculation of the Fourier transforms of the three possible identical duplex structures would allow a more quantitative evaluation of the fit to the X-ray data, but since really precise information concerning the expected dimensions and conformations of the various parts of a polynucleotide chain is lacking, the comparison of the results of such calculations with the X-ray data cannot be unequivocal at the present time. Nevertheless, we intend to make such comparisons as are possible in the near future.

It should be noted that the identical duplex permits the point-to-point alignment of two homologous macromolecules, a process which is presumed to be a prerequisite for genetic recombination. If the hereditary information of an organism resided in a single polynucleotide chain at that stage of its life-cycle at which an exchange of genetic factors occurs, then the union of two such chains into an identical duplex could be the mechanism by which corresponding genetic regions are brought into exact apposition. The significance of the identical duplex structure for the mechanism of DNA replication and genetic recombination will be discussed in more detail elsewhere.

* These studies were aided by grants from the National Science Foundation and the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

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THE OPTICAL ROTATORY DISPERSION OF SIMPLE POLYPEPTIDES. II

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In the accompanying note[1] evidence has been adduced that simple polypeptides in the α-helical configuration show anomalous optical rotatory dispersion of a specific kind. More particularly, it has been shown that the effective monomer rotations of poly-γ-benzyl-L-glutamate and of poly-α-L-glutamic acid obey the law

$$[m'] = \frac{a_0\lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}, \qquad (1)$$

where a_0 depends on the nature of the side chain attached to the helical skeleton and also on the solvent, but where b_0 ($\backsimeq -640°$) and λ_0 ($\backsimeq 2100$ Å) are the same for both polymers and substantially independent of the solvent. Before these experiments were undertaken, the importance of a structurally invariant term proportioned to $(\lambda^2 - \lambda_0^2)^{-2}$ had been predicted on theoretical grounds; by means of provisional calculations, its order of magnitude had been correctly estimated.[2]

In this note a more detailed account of the spectrum of an α-helix is given, and more thorough calculations of the optical activity are made. It is shown that the two prominent peptide bands at 1850 and 1500 Å can account quantitatively for the observed dispersive term involving b_0, λ_0. If, as seems plausible, this term does indeed owe its origin to the two peptide bands, the calculations show that poly-γ-benzyl-L-glutamate and poly-α-L-glutamic acid exist as right-handed α-helices in solvents like dimethyl formamide.

THE PEPTIDE CHROMOPHORE

The spectrum of formamide above 1220 Å shows two broad absorption bands, one with a maximum at 1717 Å ($N \rightarrow V_1$) and the other at 1345 Å ($N \rightarrow V_2$).[3] These systems appear at considerably longer wave lengths in N-dimethyl formamide, whose absorption curve above 1500 Å shows peaks at 1974 Å ($N \rightarrow V_1$) and 1621 Å ($N \rightarrow V_2$).[3] The peptides themselves have peaks at 1850 Å ($N \rightarrow V_1$),[4] which correlate well with the 1717 Å maximum for the unsubstituted amide, on the one hand, and with the 1974 Å peak of the N-disubstituted amide, on the other.[5] Their spectra at shorter wave lengths do not appear to have been measured, but it may be anticipated that the only strong band systems above 1350 Å due to the peptide linkage occur near 1480 Å ($N \rightarrow V_2$), by a similar interpolation. All these absorption bands are attributed to excitations which are predominantly localized among the four unsaturation (π) electrons of the planar $-\overset{\overset{O}{\|}}{C}-NR_1R_2$ groups.

The remaining, longer-wave-length transitions (>1350 Å) in peptides are of three kinds. Some may arise from the chromophores, such as acid or ester functions, which belong to particular side chains. Others are due to Rydberg transitions, in which an unsaturation electron is promoted to an orbital of large radius, and to $n_0 \rightarrow \pi^*$ excitations involving a nonbonding oxygen electron (n_0) and an excited unsaturation orbital (π^*). The Rydberg series are not very strong, but their sharpness in formamide enables them to be seen; the latter $n_0 \rightarrow \pi$ transitions are very weak and completely overshadowed by the two prominent $N \rightarrow V$ systems.[3]

For reasons that will appear, we shall attribute the values of the helical constants, b_0, λ_0 to the two $N \rightarrow V$ systems, at least in greater measure. It is therefore of some importance to characterize these more closely. Hunt and Simpson find that the oscillator strength of the $N \rightarrow V_1$ system at 1974 Å in dimethyl formamide is 0.237.[3] It is apparently much the same for the analogous peptide band at 1850 Å, for which Ham and Platt give the value 0.27 ± 0.07.[4] For the 1620 Å band of the N-dimethyl formamide an oscillator strength of 0.209 is found,[3] which we may presume to characterize also the 1480 Å peptide band. The polarization of these

transitions is of great interest: Peterson and Simpson have studied the dichroism of sublimation flakes of myristamide in the region of the $N \rightarrow V_1$ band.[6] From this they concluded that the most likely direction of polarization was inclined at an angle of 9.1° to the NO axis (in the sense that a parallel line through the N atom cuts the CO bond).[7] In the absence of any other pertinent data, we shall suppose the polarization of the 1850 Å bands of peptides to be similarly directed. No information about the dichroism in the shorter-wave-length region is available at present. However, from the π-electronic origin of these bands it is clear that the $N \rightarrow V_2$ system at 1480 Å must also be polarized in the plane of the peptide bond and that its dipole vector is not far from orthogonal to that of the $N \rightarrow V_1$ system.

To summarize: From the available data on amides, we infer that the peptide grouping has only two strong bands above 1350 Å. The one ($N \rightarrow V_1$), with an oscillator strength of 0.237, lies at 1850 Å and is polarized at an angle of 9.1° to the direction of the NO bond. The other ($N \rightarrow V_2$) has its maximum at 1480 Å, an oscillator strength of 0.209, and is also polarized in the plane of the peptide bond, but perpendicularly to the $N \rightarrow V_1$ system.

THE SPECTRUM OF THE HELIX

The spectrum of an α-helix will closely resemble that of the simpler peptides, with the absorption systems of the side-chain chromophores superposed on the bands enumerated above. However, the structure of the $N \rightarrow V_1$ and $N \rightarrow V_2$ systems will now be more complex. Owing to the interaction of the instantaneous electric moments of one peptide bond with those of its neighbors, these systems are both composite. The $N \rightarrow V_1$ spectrum, for example, consists of one component which is polarized parallel to the screw axis, and a degenerate pair whose polarization is perpendicular to this. In the upper state of the former, the instantaneous moments of the chromophores are all in phase. In the excited states of the latter, however, the phase difference between the electronic motions in adjacent groups is given by the angle $2\pi/P$, where $P = 3.7$ is the number of residues per unit turn of the α-helix. States in which the phase differences are different also exist—indeed, for a helix of N residues, there are $(N - 3)$ of these. But they do not appear as the upper states of band systems, since transitions to them are electronically forbidden: the parallel and perpendicular systems account for the total intensity.

For illustrative purposes only, we show these two states for the simpler helix, with $P = 4$, in Figures 1 and 2. The electric moments are drawn as the diagonal arrows in squares representing the plane of the peptide bond. The actual moments for the $N \rightarrow V_1$ system bear a close geometrical resemblance to these diagrams. For example, the planes of the peptide bonds in the α-helix are also parallel to the screw axis. The moments for the $N \rightarrow V_2$ system may be pictured by drawing the arrows along the other diagonals of their respective squares, maintaining their mutual phase relationships. Only one component of the degenerate pair of states is shown (Fig. 2); the other would be represented by turning the screw through one-quarter of its pitch.

It is clear, on comparing the two diagrams, that the energies of the respective excited states differ because of the electrostatic interactions between the different

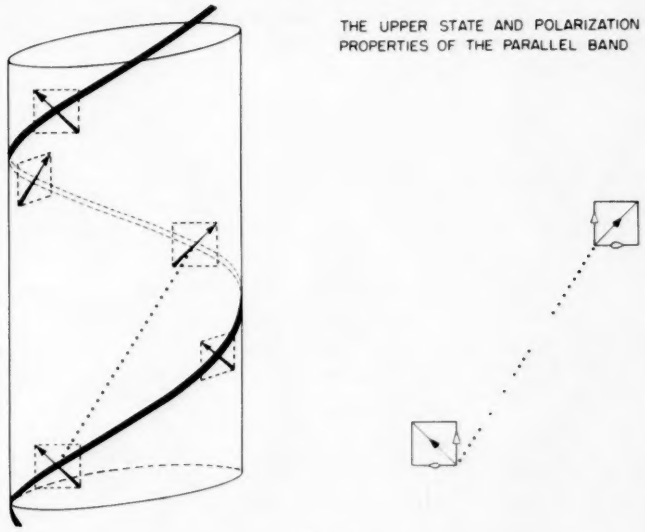
THE UPPER STATE AND POLARIZATION
PROPERTIES OF THE PARALLEL BAND

FIG. 1.

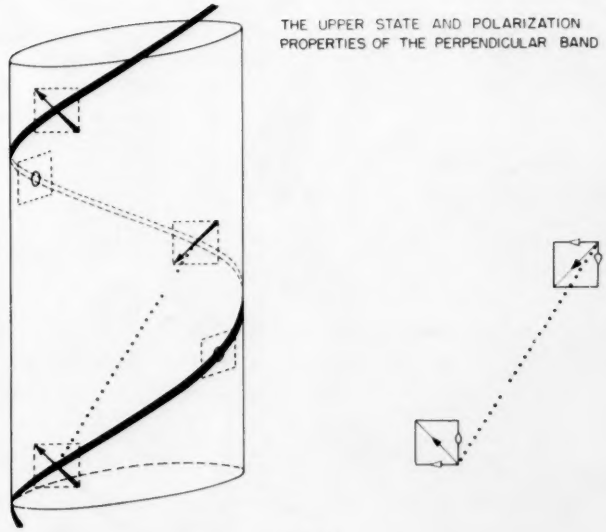
THE UPPER STATE AND POLARIZATION
PROPERTIES OF THE PERPENDICULAR BAND

FIG. 2.

residue moments. Explicitly, if $\nu_{\parallel i}$, $\nu_{\perp i}$ represent the excitation energies of the upper states for the parallel and perpendicular components of the ith band system ($N \rightarrow V_1$, say), it is easily seen that

$$\Delta\nu_i = \nu_{\parallel i} - \nu_{\perp i} = \sum_l \left[1 - \cos\left(\frac{2\pi l}{P}\right)\right] \frac{V^i{}_l}{hc} \tag{2}$$

where the summation is taken over all neighbors l of a given residue; $V^i{}_l$ is the energy of interaction between the instantaneous moments of this residue with those of the lth; it is assumed that the helix is long, and $\Delta\nu_i$ is expressed in wave numbers.[1]

The instantaneous residue moments are not point dipoles but, rather, extended over the OCN atoms composing this particular chromophore—for it is along this path that the chromophoric electrons may be supposed to move. In so far as they interact with electromagnetic waves, they may be treated as point dipoles, but their multipolar character will manifest itself in their interactions with near neighbors.

The diagrams are useful not only for picturing the origin of the terms separating the different upper states within a given composite band system of this kind. Vector addition of the arrows, treated now as point dipoles, is easily shown to represent, both in magnitude and direction, the dipole vector whose interaction with radiation gives rise to the transitions: in Figure 1 it is obvious that the electric vector of the light must be parallel to the screw axis, and, similarly, transitions to the state pictured in Figure 2 must be perpendicularly polarized. (It is because these vector sums vanish by cancellation that states, whose residue moments have different phase relationships, do not appear in the spectrum.) Again, when the electrons are accelerated by radiation near the resonant frequency, their resultant momentum at any instant may be represented by the same vector sum. We see, in particular, that perpendicularly polarized light will induce electronic motions with a net angular momentum parallel to the polarization vector and, *mutatis mutandis*, that when the electric vector vibrates along the screw axis, it will give rise to fluctuating electronic angular momenta about this axis. (This is more obvious, in both figures, if the vectors are compounded in pairs—that of the first residue with that of the third, and that of the second with that of the fourth.) These angular momenta are of course associated with magnetic moments, by Ampère's law.

Now the condition that a given electronic transition be optically active is just that there should be a nonvanishing magnetic moment parallel to the electronic displacements induced by the radiation. Both the parallel and perpendicular components of such composite band systems satisfy this criterion in first order. Neglecting, for the moment, any other sources of asymmetry that these chromophores may have, it may be shown that their rotational strengths are given by[1]

$$r_{\parallel i} = \frac{3e^2h}{8\pi\, mc} f_i \rho_i e_{it} e_{iv} = -r_{\perp i}, \tag{3}$$

where f_i is the oscillator strength of the complete band, per residue, ρ_i is the distance from the screw axis of the moment vector, whose tangential and vertical direction cosines are e_{it}, e_{iv}, respectively. (This applies to a right-handed helix;

the sign of the second term is reversed for a left-handed helix. The terms, vertical and tangential, refer to a cylindrical co-ordinate system, in which the outward radial vector, the tangential vector, and the vertical direction of the advancing screw form a right-handed Cartesian set of axes.) These are both exceedingly large, but opposed. Their contributions to the residue rotation at wave lengths far from resonance cancel apart from terms proportional to the separation $\Delta\nu_i$ of the two component systems in the ith band. (However, if one could arrange for the incident light to propagate along the screw axis alone, the electric vector would always be perpendicularly polarized, and the observed rotations would be very high.) In terms of the quantities b_i introduced in the accompanying note, we see that they are, however, responsible for the abnormally dispersing term $b_i\nu_i^2\nu^2/(\nu_i^2 - \nu^2)^2$, where $\nu_i = {}^1/_2(\nu_{\parallel i} + \nu_{\perp i})$ and

$$b_i = -\frac{72Ne^2}{mc^2} f_i\rho_i e_{it}e_{iv}\left(\frac{\Delta\nu_i}{\nu_i}\right). \tag{4}$$

This angle will be appreciable only if the moment vector for the appropriate transition has nonvanishing components along and tangentially to the screw axis, for otherwise $e_{it}e_{iv}$ vanishes. Since $\Delta\nu_i$, a measure of the interactions between the residue moments, is large only when the associated transition is strong, b_i is roughly proportional to the square of the oscillator strength f_i. For the left-handed helix the sign is naturally reversed.

The $n_0 \rightarrow \pi^*$ bands will show no detectable dispersion of this sort, since their oscillator strengths will be less than 0.001. On similar grounds, the Rydberg transitions may also be neglected in this respect. The side-chain chromophores may absorb intensely but will probably rotate more or less freely in solution, which tends to average out the terms contributing to $\Delta\nu_i$; in addition, these are a long way apart from each other, so that they will interact but feebly on this score also. Thus, of the bands at wave lengths greater than 1350 Å, only the $N \rightarrow V_1$ and $N \rightarrow V_2$ systems should show complex dispersion of this kind. The other bands will be optically active, but their partial rotations are represented by the simple dispersive terms $a_i\nu^2/(\nu_i^2 - \nu^2)$ and do not contain additional terms involving b_i's of any size. Since the successive peptide chromophores, of which the basic α-helical skeleton is composed, are essentially unaffected by variations in solvent, temperature, and side chain, provided only that they do not destroy the α-helix, we see that the only important b_i terms should also be independent of such variations. This is not true, of course, for the a_i terms.

THE CALCULATION OF $\Delta\nu_1$ AND $\Delta\nu_2$

We shall now evaluate the quantities on the right-hand side of equation (4) for the two strong peptide bands, using the assignments discussed above. By elementary geometry,

$$\begin{aligned} \rho_1 &= 1.59\ \text{Å} = \rho_2, \\ e_{1t}e_{1v} &= -0.493 = -e_{2t}e_{2v}, \\ f_1 &= 0.237, \qquad f_2 = 0.209, \\ \nu_1 &= 54{,}050\ \text{cm}^{-1}, \qquad \nu_2 = 67{,}570\ \text{cm}^{-1}, \end{aligned} \tag{5}$$

so that we only have to evaluate $\Delta\nu_1$ and $\Delta\nu_2$. (The subscripts 1 and 2 refer, respectively, to the $N \rightarrow V_1$ and $N \rightarrow V_2$ systems.)

Consider one particular term V_l^i of equation (2), which represents the interaction of the electric moments induced by the ith band in the lth residue and in a given residue, m, say. More completely,

$$V_{lm}^{\ i} = \int\int \psi_{il}^*\psi_{0m}^*\left(\sum_s\sum_t \frac{e^2}{r_{l_s m_t}}\right)\psi_{0l}\psi_{im}\, d\tau_l\, d\tau_m, \tag{6}$$

where $e^2/r_{l_s m_t}$ represents the electrostatic repulsion between an electron l_s of the lth residue and another m_t of the mth; ψ_{0l} and ψ_{il} are the wave functions of the former electrons in their ground and ith excited states, respectively. The evaluation of this integral is standard if we are prepared to replace the electric moments by point dipoles. However, since the extension of a particular (conjugated) chromophore is of the same order of magnitude as its separation from its immediate neighbors, this approximation is invalid (it leads to errors of about ± 50 per cent). In its place we adopt a method of calculation which is based on approximations suggested by current electronic theories of spectra.

We regard the chromophoric electrons of a given residue as represented by wave functions made up from antisymmetrized products of atomic orbitals. It is unnecessary to specify in what linear combinations these occur; they may be constructed as molecular orbital functions with configurational interaction, for example. We shall also suppose the four chromophoric electrons to belong only to the three conjugated atoms of the peptide bond, the overlap between whose π-orbitals we neglect. (It has been shown, for example by Parr,[8] that the latter approximation is very good so far as electrostatic interactions of this kind are concerned.) Now consider the representation of an arbitrary "one-electron" operator

$$\mathcal{O}_l = \sum_s \mathcal{O}_{l_s} \tag{7}$$

in this scheme, where $\mathcal{O}_{l_s}$ acts on the co-ordinates of electron l_s but on no others of the lth residue. It is readily demonstrated that

$$\begin{aligned} \int \psi_{il}^*\, \mathcal{O}_l\psi_{0l}\, d\tau_l &= C_O^{\ i}\varpi_{O_l} + C_C^{\ i}\varpi_{C_l} + C_N^{\ i}\varpi_{N_l}, \\ \varpi_{R_l} &= \int \phi_{R_l}^*(l_s)\mathcal{O}_{l_s}\phi_{R_l}(l_s)\, d\tau_{l_s} \qquad (R = \mathrm{O, C, N}), \end{aligned} \tag{8}$$

ϕ_{R_l} being a normalized $2p\pi$ orbital of atom R in the lth residue. The coefficients $C_R^{\ i}$ are independent of the specific nature of the operator $\mathcal{O}$. In particular, setting $\mathcal{O}_l$ equal to the identity operator, we find that

$$C_O^{\ i} + C_C^{\ i} + C_N^{\ i} = 0, \tag{9}$$

since ψ_{il}, ψ_{0l} are orthogonal. Again, setting $\mathcal{O}_l = \vec{\mu}_l$, the electric dipole vector for the electrons of the lth chromophore corresponding to this transition, we have

$$\vec{\mu}_{il} = e(C_O^{\ i}\vec{r}_{O_l} + C_C^{\ i}\vec{r}_{C_l} + C_N^{\ i}\vec{r}_{N_l}), \tag{10}$$

$\vec{r}_{R_l}$ being the position vector of atom R_l. Now $\vec{\mu}_{il}$ is determined, apart from a phase factor, by dichroic studies. Thus, since the three conjugated atoms are not colinear, equations (9) and (10) together determine C_O, C_C, and C_N apart from a

common phase factor (whose choice is in any case immaterial); for convenience we suppose the C_R to be real. We now set $\mathcal{O}_{l_s} = e^2/r_{l_s m_t}$ and apply the same reasoning to the mth residue, finally finding that

$$V_{lm}{}^{i} = \sum_R \sum_S C_R{}^{i} C_S{}^{i} \int\int |\phi_{R_l}(l_s)|^2 \frac{e^2}{r_{l_s m_t}} |\phi_{S_m}(m_t)|^2 \, d\tau_{l_s} \, d\tau_{m_t}. \tag{11}$$

The distance between two atoms in different residues is large enough, with respect to their respective radii, that we now replace these integrals by their asymptotic values, namely, $e^2/r_{R_l S_m}$, the energy of repulsion between a charge e on nucleus R_l and a like charge situated at nucleus S_m. If the directions of polarization as well as the oscillator strengths of transitions are known, the procedure we use has the merit of dispensing with the dipole approximation, without requiring a knowledge of those higher multipolar terms that spectroscopic studies do not yield.

The method outlined was followed in order to estimate the interaction of a given residue with its six successors and six predecessors in the helical sequence. The usual dipole approximation is valid thereafter and was used for the next eight residues in both directions. The contribution of more distant terms was estimated using an asymptotic integral. (It was found that the first three neighbors on either side of a given residue account for 70 per cent or more of the final sums.) The values we obtained were

$$-\Delta\nu_1 = 2800 \text{ cm}^{-1}, \qquad -\Delta\nu_2 = 3130 \text{ cm}^{-1}. \tag{12}$$

Combining these with equation (5) and substituting in equation (4), we obtain the angles

$$b_1 = -1774^\circ, \qquad b_2 = +926^\circ, \tag{13}$$

for a right-handed α-helix. These are the best values that we could obtain on the basis of the available spectroscopic information.

THE OPTICAL ROTATORY DISPERSION

It should be emphasized that the b_i's we have just calculated are both almost maximal. The oscillator strengths of the bands are high, the residue chromophores interact strongly, and the directions of polarization give orientational factors $|e_t e_v|$ close to $^1/_2$, which they cannot, of course, exceed. The concomitant bands are also the only systems above 1350 Å showing these features. This suggests that these two terms dominate the dispersive form of the optical rotatory power. Bands at shorter wave lengths are not expected to influence the observed rotations between 7000 and 3000 Å to anything like the same extent (see below). Accordingly, we shall attribute the values of b_0 and λ_0 in equation (1) to the contributions from b_1, b_2 and λ_1, λ_2 alone:

$$b_0\lambda_0{}^6 = b_1\lambda_1{}^6 + b_2\lambda_2{}^6, \qquad b_0\lambda_0{}^4 = b_1\lambda_1{}^4 + b_2\lambda_2{}^4. \tag{14}$$

Some empirical support is found for this assumption, inasmuch as b_0, λ_0 are apparently helical invariants, whereas a_0 is not. This is most easily rationalized on the basis of equations (11) of the preceding note; if the terms containing a_t are relatively small in the second and third of these, they reduce to their successors (12).

In the latter, b_0, λ_0 are now determined by the b_i, λ_i alone, and, as we have shown, these should depend only on the nature of the helical skeleton. The parameter a_0 is then solely responsible for the variable dispersive features, as observed.

Substituting the values we have computed, it is found that

$$b_0 = -580°, \qquad \lambda_0 = 2000 \text{ Å}, \tag{15}$$

for a right-handed α-helix. This is embarrassingly close to the observed values of about $-640°$ and 2100 Å, respectively. For a left-handed α-helix, the predicted sign for b_0 is simply reversed. It is tempting, therefore, to regard this correspondence as meaningful and to conclude that both the helices we have studied are right-handed. Before so doing, however, it is well to review the assumptions underlying this comparison.

The only data we have used in these calculations are those concerning the dichroism of the two peptide bands. We can justly claim that the numbers we derived from the available information were the only sensible ones. Thus our values for b_1, b_2, λ_1, and λ_2 are likely to be as accurate as the data on which they are based. However, it would be preferable to use spectral studies of the α-helix itself (polyalanine would be the most suitable material). Working with oriented samples, it should be possible to obtain $\Delta\nu_1$, $\Delta\nu_2$ and $|e_{1l}\ e_{1v}|$, $|e_{2l}\ e_{2v}|$ and f_1, f_2 directly. These would enable us to calculate b_1, b_2 with the least uncertainty, and, at the same time, they afford an internal check of our method for computing $\Delta\nu_1$, $\Delta\nu_2$. But, owing to the diffuse nature of the bands, it is unlikely that the latter can be measured very accurately.

Certainly the most serious assumption we have made is that b_0, λ_0 are determined by b_1, λ_1 and b_2, λ_2 through equations (14). This can be justified only heuristically. If further measurements on simple polypeptides with different side chains confirm the constancy of b_0, λ_0, but the variability of a_0, we shall be on firmer ground. For this will show, on empirical grounds, that equations (12) of the preceding note are at least approximately valid. It then only remains to justify our neglect of the bands lying at shorter wave lengths than 1350 Å. In this connection we note that, although the values of b_0, λ_0 which we have calculated differ appreciably from b_1, λ_1, the actual rotations at important wave lengths (e.g., 5000–3500 Å), calculated with both sets of parameters, do not differ nearly as much. This means that the presence of the 1480 Å band modifies, but does not greatly affect, the abnormal dispersion due to the 1850 Å system. Although the 1480 Å system has a value of b that is absolutely large—one that is unlikely to be exceeded by the shorter-wave-length systems, which correspond to more localized, if more highly excited oscillators—the adverse λ_i dependence reduces its importance very considerably. On these grounds, the influence of the shorter-wave-length systems is likely to be yet smaller; its neglect should not seriously affect our conclusions.

Our analysis is therefore only tentative. However, it suggests the following experiment. If it were possible to observe the optical rotation for light around 3500 Å, propagating entirely along the length of the helices, we should expect them to be strongly dextrorotatory on the basis of equation (3), since the electric vector is now always perpendicular to the screw axis. The measurement is hardly straightforward. For the molecules need to be well aligned in a medium of similar

refractive index, which permits the side chains to rotate unless these have no strong chromophores of their own.

DISCUSSION

It will be noticed that we have not attempted to calculate the actual rotation at any wave length, for this involves a_0 in addition to b_0, λ_0. We do not believe that it is possible at the present time to make reliable computations of a_0. Indeed, our less ambitious identification of b_0, λ_0, through equations (14), with b_1, λ_1 and b_2, λ_2 can be only tentative until it is confirmed by more direct dichroic studies of the polypeptides themselves. Fitts and Kirkwood are more optimistic and have recently claimed to assess the rotation itself, due to a right-handed α-helix with no asymmetric α carbon atoms ("polyglycine").[9] Even more recently,[10] they have compared their results with data on the differences in specific rotation between the α-helical and randomly coiled forms of poly-L-peptides. It seems appropriate, therefore, to discuss the difficulties that we consider to be inherent in their work. In order to avoid a more general discussion of optical activity, we shall confine our remarks to their application of Kirkwood's own polarizability theory to the present problem.[11, 12]

1. *The α-Helix Is Treated as a Continuous Uniform Thread.*—Since there are only 3.7 operative units—whose intrinsic properties are highly unsymmetrical—per turn of the helix, their model cannot at best be other than a most crude facsimile of a real polypeptide.

2. *The Thread Is Regarded as Cylindrically Symmetric, Locally.*—The polarizability tensors of its infinitesimal sections are assumed to be ellipsoids of revolution, with their principal axes along the respective directions of the thread. If a continuum model of the successive peptide linkages is to be adopted at all, surely it must be acknowledged that the polarizability tensor has much lower symmetry than this. The planes of the peptide bonds are parallel to the screw axis and contain the segments of the helical thread. Fitts and Kirkwood imply that peptides are no more polarizable along the conjugated NCO path, in a direction almost coincident with the polarization of the first strong $N \rightarrow V_1$ band, than they are along an axis perpendicular to this plane. All measurements on the anisotropies of conjugated systems refute this assumption. Indeed, it would be much more reasonable to assume that, if the individual segments were to be described by polarizability tensors with this symmetry, their principal axes were normal to the thread directions and to the outward radius vectors from the screw axis.

It is interesting to note that the consequences of this alternative assumption expose the weaknesses of the continuum model very strikingly. In place of the $f(\theta)$ obtained by Fitts and Kirkwood—a function which behaves like $\frac{1}{6}\theta(1 + \gamma^2)^{-1/2}$ for small values of θ and is therefore integrable between 0 and θ_L—we now find a function which behaves like $\theta^{-1}(1 + \gamma^2)^{1/2}$ near the origin: the optical activity becomes logarithmically infinite. And this singularity can be removed only by adding an artificial cutoff, which is foreign to the premises of the model.

3. *The Anisotropy Ratio β Is Taken To Be about $^1/_3$ for the Glycine Residue.*—We can find no basis for this supposition. A glycine residue has an approximate plane of symmetry, coinciding with that of the peptide bond, but no other elements of symmetry. Thus the orientation of the principal axes of the polarizability tensor

for such a group is frequency dependent, in the first place, and, as we have remarked, there is no reason why two of the axes should be equivalent: β is not a well defined scalar at all, far less equal to $^1/_3$, and yet the results of Fitts and Kirkwood are directly proportional to its square.

4. *It Is Assumed that the Contribution to the Rotation from the Asymmetric α Carbon Atoms Is the Same Both for the α-Helical and the Randomly Coiled Configuration.*—It is on this basis that Fitts and Kirkwood compare their calculations with experimental data. If the groups directly attached to the α carbon atoms were rotating freely, the optical activity of the randomly coiled forms would vanish identically. Indeed, it is to the restricted rotation about bonds linking the various groups to asymmetric carbon atoms that Kirkwood, in his 1937 paper, attributes the rotatory power of such systems.[11] The assumption made by Fitts and Kirkwood therefore implies that the conditions for restricted rotation about the bonds to the asymmetric α carbon atoms are identical both in the randomly coiled and in the α-helical forms of the polypeptides. But it is in just this respect that, by definition, the two configurations differ. The comparison which they make between theory and experiment is without significance.

5. *The Dispersion Described by Fitts and Kirkwood Is Contradicted by the Observed Behavior.*—Fitts and Kirkwood remark that their model also leads to an abnormal frequency dependence of the form we had previously predicted. On the grounds already stated, we cannot admit that their formulation of the dispersive behavior is well founded. Their result requires that the helical contribution, both to a_0 and to b_0, should have the same sign, whereas they are observed to differ.

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[12] Although our work has much in common with the polarizability theory, we do not agree with certain important premises of the latter.

*SYNERGISM OF TWO CYTOPLASMICALLY INHERITED MUTANTS IN NEUROSPORA CRASSA**

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The complementarity of nonallelic nuclear genes in *Neurospora* heterokaryons has been recognized since the early work of Dodge[1] and Beadle and Coonradt,[2] but little is known of interactions between cytoplasmic mutants. The mutations of the particle-bound respiratory system in *Neurospora* offer an opportunity for such a study. The first of these maternally inherited cytoplasmic mutants, mi-1 (*poky*) and mi-3, were reported by Mitchell and Mitchell[3] and Mitchell *et al.*[4] and are characterized by subnormal growth rates and defective cytochrome systems. Biochemical and genetic tests have shown that the two mutants are not identical;[4–8] hence it seemed possible that a cytoplasmic mixture of *poky* and mi-3, a heterocytosome, might restore the normal phenotype. No improvement of the growth rate was observed in mixed cultures, however.[4]

More recently in this laboratory another cytoplasmically inherited mutant, mi-4, has been found that phenotypically resembles *poky*. Both mi-4 and *poky* are transmissible to other strains by heterokaryosis; hence their genetic basis is not merely the absence of some normal cytoplasmic entity but rather the presence of an abnormal one. Although a detailed biochemical analysis of mi-4 has not yet been completed, a difference between mi-4 and *poky* is apparent because they interact in a heterocytosome to produce a phenotype different from either strain alone. The experiments reported here show that an essentially normal growth rate results from cytoplasmic fusion of these two slowly growing strains and that the stimulation cannot be attributed to nuclear interaction.

Description of the Strains.—The spectroscopic appearance of mycelial pads of mi-4 is indistinguishable from that of *poky*[4] when a small grating spectroscope is used; possibly a more refined technique would reveal differences. The rate of oxidation of reduced cytochrome c in the presence of mycelial homogenates, measured with a Beckman spectrophotometer, is similar for mi-4 and *poky* and markedly retarded in comparison to normal strains.

When mi-4 is the conidial parent in crosses, its phenotype is not transmitted, although associated nuclear markers segregate normally. In the reciprocal cross, fertile perithecia have never been produced; thus the maternal inheritance of mi-4 is an extrapolation from its other genetic properties. Fertile crosses have been obtained, however, when the heterocytosome of mi-4 + *poky* was the protoperithecial parent in crosses with either *poky* or wild type. When *poky* was the male parent, all the progeny (*ca.* 10,000 ascospores examined) were phenotypically mutant, but when a wild type was the male parent, up to 1 per cent of the progeny were phenotypically wild; however, this was in the range encountered in similar experiments of *poky* × wild crosses previously reported.[3] Mi-4 arose spontaneously in a lysine-requiring strain (4545)[9] and has been obtained in other genetic backgrounds only by heterokaryosis. Heterokaryons between *lys*, mi-4 and stocks with wild-type cytoplasm have shown the mi-4 phenotype after a variable

period of normal growth. From such heterokaryons, homokaryotic isolates have been obtained free of *lys* nuclei but having the mi-4 cytosome.

The *lys*, mi-4 strain in horizontal growth tubes shows a prolonged lag, then a period of growth at subnormal rates, and eventual cessation of growth. If growth resumes, it again ceases after a variable interval. The cycle of stopping and starting may be repeated several times. Similar cycles are observed with *poky* strains, but the reason for the cyclic behavior remains unknown.

In addition to the rate and duration of uninterrupted growth in horizontal tubes, the growth of the conidia on sorbose medium, used to induce colonial-type growth, has been one of the most useful criteria for recognizing the mi-4 and *poky* phenotypes. When mi-4 conidia are plated on sorbose medium, the majority do not grow, but some produce microcolonies that, except for their mycelial morphology, are reminiscent of the petite colonies of yeast.[10] A high proportion of *poky* conidia are viable, but these, too, produce microcolonies. The mi-4 microcolonies stop growing when small and are seldom viable thereafter, even when transferred to regular medium. The *poky* microcolonies continue to grow slowly, may eventually reach normal size, and are usually viable on transfer. It is not always possible to distinguish *poky* and mi-4 phenotypes from each other, since the degree of expression is influenced by the nuclear genome, but both can always be clearly distinguished from wild type.

For use in the present experiments a *poky* albino, pantothenate-requiring strain (mi-1, 4637, 5531, A)[4, 9] that is heterokaryon positive with *lys*, mi-4, A was selected from a cross of 3627-2a(*poky*)[8] × *pan*, *al*-1, A. This strain, designated "*pan*, *poky*" for this discussion, shows the behavior just described for *poky*. From the *lys*, mi-4 and *pan*, *poky* strains, two other strains were derived, having the *lys* and *pan* (nuclear) genetic background but having normal cytoplasm. The method of obtaining these strains was suggested by the observation that the conidia from newly formed heterokaryons between mi-4 or *poky* and normal strains, taken from a point close to the mixed inoculum, would yield some normal colonies on sorbose agar. Accordingly, controlled inocula were prepared (Pittenger *et al.*)[11] with *lys*, mi-4 or *pan*, *poky* as the minor component and a normal strain with different nuclear markers as the major component, to provide a large admixture of normal cytoplasm. On plating conidia from the resultant heterokaryon, a few normal colonies were obtained that were homokaryotic for *lys* and *pan*. The wild-type condition of the cytoplasm of these isolates was confirmed by growth studies, conidial plating, and spectroscopic examination.

The evidence for synergism of mi-4 and *poky* is the behavior of a number of combinations of the three cytosomes, normal, mi-4, and *poky* and two nuclear types, *lys* and *pan*. The characteristics of these combinations are summarized in Table 1, and more detailed descriptions are given in the sections that follow.

The lys, mi-4 + pan, poky Combination.—This combination was prepared from mixed inoculum[11] and transferred to horizontal growth tubes containing minimal agar supplemented with optimal lysine and calcium pantothenate. The growth rates of the combination and the original strains were measured by the method of Ryan *et al.*[12] at 30° C. As shown in Figure 1, the heterokaryon (and heterocytosome) grows much more rapidly than the component strains. After completion of growth, conidia from the ends of the growth tubes were plated on minimal and

supplemented sorbose medium. A majority of the colonies from the heterocytosome were of normal size on all media within a week after plating, whereas conidia of the two original strains formed only microcolonies in the same period of time. Growth of the heterokaryon and the two homokaryons was compared by growing

TABLE 1

SUMMARY OF THE BEHAVIOR OF A NUMBER OF COMBINATIONS OF THREE CYTOSOMES, NORMAL, mi-4, AND *poky*, AND TWO NUCLEAR TYPES, *pan* AND *lys*

GENOTYPE		PHENOTYPE*	STABILITY OF PHENOTYPE
Nucleus	Cytosome		
pan	*poky*	Mutant	Stable
lys	*poky*	Mutant	Stable
pan + *lys*	*poky*	Mutant	Stable
pan	mi-4	Mutant	Stable
lys	mi-4	Mutant	Stable
pan + *lys*	mi-4	Mutant	Stable
pan	mi-4 + *poky*	Wild	Becomes mutant
lys	mi-4 + *poky*	Wild	Becomes mutant
pan + *lys*	mi-4 + *poky*	Wild	Becomes mutant
pan	Normal	Wild	Stable
lys	Normal	Wild	Stable
pan + *lys*	Normal	Wild	Stable
pan + *lys*	Normal + mi-4	Wild	Becomes mutant
pan + *lys*	Normal + *poky*	Wild	Becomes mutant

* Phenotype refers to initial growth rate and ability of conidia to form normal colonies on sorbose medium but not to spectroscopic analysis.

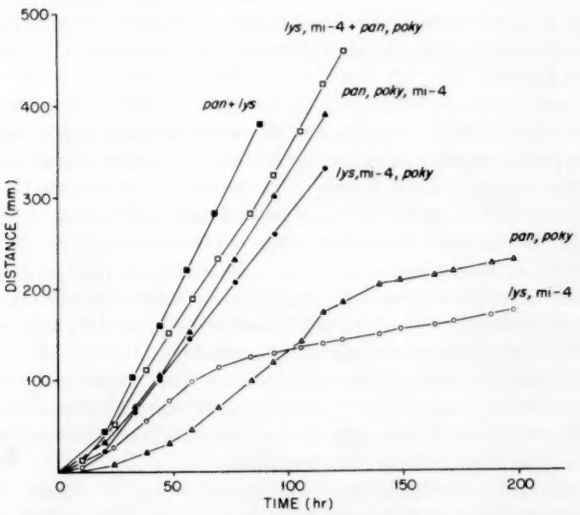

FIG. 1.—Characteristic growth curves on supplemented medium of identical nuclear types, *pan* and *lys*, with various combinations of normal, mi-4, and *poky* cytosomes.

the cultures in supplemented liquid medium for 3 days at 30° C. Comparison of the dry weights of the mycelial pads showed that the heterokaryons had grown more than $2^1/_2$ times as much as the average of the two homokaryons. Despite the striking stimulation of growth, the spectroscopic appearance of mycelial pads of

the heterocytosome was always mutant. Although the heterokaryons may have normal growth rates over distances of 1,000 mm. or more, the normal growth is not permanent, and eventually the stopping and starting phenomenon characteristic of mi-4 or *poky* was also observed in the heterokaryons.

Combinations in Which Both of the Strains Have Normal Cytoplasm.—Dodge[1] and Dodge *et al.*[13] have demonstrated with *N. tetrasperma* that the heterokaryotic vigor observed when two slowly growing strains were combined can be caused by the interaction of genes located in different haploid nuclei of the heterokaryon. Consequently, where a synergistic effect is noted and when the cytoplasm is suspected of having an effect, it must first be shown that nuclear interaction is not the primary cause of the observed results. That nuclear interaction alone could not account for the results in the *lys*, mi-4 + *pan*, *poky* heterokaryon (and heterocytosome) was indicated by the results of similar experiments in which strains of the same nuclear constitution were used, but with the mi-4 and *poky* cytoplasm replaced by normal cytoplasm. In marked contrast to the results just presented, the growth rate of the heterokaryon (Fig. 1) with the normal cytoplasm was no greater than that of the faster-growing component.

Combinations in Which One of the Strains Has Normal Cytoplasm.—Heterokaryons in which one of the component strains has normal cytoplasm, e.g., *lys* + *pan*, *poky* or *lys*, mi-4 + *pan*, initially show normal growth, and the conidia produce normal colonies, but such cultures eventually show the mutant phenotype as expressed by the formation of microcolonies and abnormal growth behavior. The production of conidia that showed the mutant phenotype on plating considerably preceded the retardation in growth.

Lys, mi-4, poky and pan, mi-4, poky Combinations.—Although these experiments indicate that the increased growth rate of the heterokaryons with the heterocytosome was not the result of any nuclear interaction, the most conclusive evidence is the fact that from *lys*, mi-4 + *pan*, *poky* cultures it is possible to isolate from conidia homokaryotic cultures of *lys* and *pan* whose growth rates were nearly wild type (Fig. 1). Where only one nuclear type is present, the increased growth rate must be attributed to the heterocytosome. It should be pointed out that when either homokaryotic or heterokaryotic conidia are isolated from such heterokaryotic cultures and subcultured, the resultant strains may have different growth rates, ranging from essentially normal to those comparable to the original mutant strains. One possible explanation is that the distribution of the two mutant constituents in the conidia results in a range of proportions only some of which are capable of giving normal growth. All such isolates are, however, unstable and revert to the mutant phenotype after prolonged propagation.

Heterokaryotic poky Combinations.—The growth rates of various *poky* heterokaryons as compared to mi-4 + *poky* heterokaryons were those to be expected on the basis of the hypothesis that the observed synergism is primarily the result of the heterocytosome. In Table 2 are given the fastest growth rates achieved on supplemented medium by five homokaryotic strains (all heterokaryon positive) derived from different isolations in addition to several heterokaryons between different *poky* strains and between mi-4 and *poky* strains. None of the growth rates listed for the *poky* heterokaryons persisted for more than 36–48 hours, after which the growth rates were much slower. However, the growth rates of the mi-4 + *poky* hetero-

karyons were constant throughout the 350-mm.-long growth tubes. Although some of the *poky* heterokaryons grew slightly faster than either of the component strains alone, this heterokaryotic vigor was not nearly so great as the synergism shown by the mi-4 + *poky* cultures.

TABLE 2

FASTEST GROWTH RATE ACHIEVED ON SUPPLEMENTED MEDIUM AT 30° C. BY DIFFERENT CYTOPLASMIC AND NUCLEAR COMBINATIONS

Strain	Growth Rate (Mm/Hr)	Strain	Growth Rate (Mm/Hr)
pan, *poky* No. 14	2.2	*pan*, *poky* No. 14 + *pan*, *poky* No. 17	2.4
pan, *poky* No. 16	1.7	*pan*, *poky* No. 16 + *pan*, *poky* No. 17	1.9
pan, *poky* No. 17	2.2	*pan*, *poky* No. 17 + *lys*, *poky* No. 8	2.8
lys, *poky* No. 8	1.6	*pan*, *poky* No. 17 + *lys*, mi-4	4.5
lys, mi-4	2.3	*lys*, mi-4 + *lys*, *poky* No. 8	5.0
pan, *poky* No. 14 + *pan*, *poky* No. 16	2.9		

Discussion.– From the evidence presented here, there can be little doubt that the observed synergistic effect on growth is caused by the coexistence of mi-4 and *poky* cytoplasm. The results suggest that in the cytoplasm of wild-type *Neurospora* some cytoplasmic constituent has separate genetic continuity and that *poky* and mi-4 represent different alterations or mutations of this constituent. No critical evidence is at hand to identify this constituent with the particle bearing the terminal oxidase system. Neither mi-4 nor *poky* is the result of an interaction between the nucleus and the normal cytoplasm, since both nuclear types isolated from mi-4 and *poky* cultures will maintain a normal cytoplasmic condition indefinitely. The cytoplasmic elements characteristic of mi-4 and *poky* appear to have their own genetic continuity and are capable, under the conditions of the experiments, of replacing their wild-type analogue. A competitive replacement of wild type by mi-4 or *poky* may account for the instability of the heterocytosome in which either mutant cytoplasm initially coexists with the wild type. A similar competition between mi-4 and *poky* may account for the instability of the heterocytosome and for the failure to find evidence for the transmission of the heterocytosome by crossing.

The synergistic action of the mutants could be explained in one of two ways: either mi-4 and *poky* have complementary action, or they can co-operate to form the normal cytoplasmic constituent. No evidence for normal cytoplasm in the heterocytosome has yet been found, since no stable normal strains have appeared among conidial isolates, even under the same conditions that allow recovery of stable normal strains from cultures with mixed mutant and wild-type cytoplasm. Similarly, when the stimulated heterocytosome is the protoperithecial parent in crosses with *poky*, no wild-type ascospores are recovered. The spectroscopic appearance of the stimulated heterocytosome is mutant, whereas a wild-type appearance would be expected if normal particles were present in large numbers. The interaction of different particles that remain separate is not without precedent, since Tissieres[14] has shown that particles from wild-type *Neurospora*, altered by treatment at alkaline pH, can reconstruct succinate oxidation in vitro when mixed with *poky* particles. An explanation for the synergistic interaction of mi-4 and *poky* must await a detailed comparison of the respiratory systems of the various cytosomes.

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*MUTATION RATES OF SEVERAL GENE LOCI IN NEUROSPORA**

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The mutation-rate studies of Stadler [1–3] led him to conclude that genes, with relatively few exceptions, are highly stable. For the *R*, *I*, and *Pr* loci in corn, he reported spontaneous mutation rates of 4.92, 1.06, and 0.11 per 10^4 female gametes, respectively, while the rates for other loci (*Su*, *C*, *Y*, and *Sh*) varied slightly from 0.01 per 10^4 female gametes. Of 1.5×10^6 gametes, he observed no spontaneous mutations of the *Wx* locus.[4] The *R* locus was regarded as "Mutable," and the more stable loci were taken to represent rather normal gene stability.

Singleton[5] recently reported quite different mutation rates for many of the same loci in corn. He reported spontaneous mutation rates of 5.8, 4.4, 5.8, and 1.4 per 10^4 male gametes for the *Pr*, *Sh*, *R*, and *Su* loci, respectively. Singleton attributed the discrepancy between his and Stadler's data to the fact that gene stability depends upon the metabolic state of the plant and also to the hypothesis that there is less selection against certain types of mutation in the male gemetophyte.

Mutation rates have been studied in many organisms. Timofeeff-Ressovsky, quoted by Dobzhansky,[6] reported spontaneous mutation rates that varied from zero and near zero to 5.15 mutations per 10^4 chromosomes for eye-color alleles in *Drosophila*. The spontaneous mutation rates for several genes of man were estimated to vary from 0.1 to 0.8 mutations per 10^4 gametes.[7] In bacteria the spontaneous reverse mutation rates varied from 0.07 to 5.61 mutations per

10^9 bacteria,[8] and in *Neurospora*, reverse, spontaneous mutation rates varied from less than 0.01 to 0.158 mutations per 10^4 conidia.[9]

Mutation-rate studies per se are confronted with severe limitations as attempts are made to characterize more fully the mutation event. As Stadler[10] pointed out, the dilemma of characterizing gene mutation awaits the development of more sensitive criteria for identifying specific mutations. At present, geneticists lack dependable criteria for distinguishing gene alteration, gene loss, and position effects. In addition, insight into the interaction among genes, provided by studies of pseudoalleles, position effects, and mutator genes, as well as the effect of metabolism on gene stability, has tended to lessen the concern given previously to the study of discrete gene mutation rates.

The present study has not circumvented the weak points of the general mutation-rate experiment; however, it has provided a methodology which may eventually lead to more revealing experimentation. The methodology permits, for the first time, a study of spontaneous and induced mutation rates of prototrophic strains of *Neurospora*.

EXPERIMENTAL

General.—The mutant strains were recovered by the filtration and selective plating technique.[11] Minor modifications of the original procedure have been employed: e.g., agitation was facilitated by a mechanical shaker rather than by forced aeration; the conidial populations were suspended in 250 ml., rather than in 500 ml., minimal medium; and incubation was shortened from 3 or more days to approximately 30 hours (X-rays appear to stimulate the rate of germination and early growth[12]).

The macroconidial strain 74A of *N. crassa* was used as the stock culture. Prior to conidial harvest, the stocks were kept on minimal agar slants for approximately 120 hours at 25° C. Media used in these experiments were the same as those reported earlier.[11] The X-ray source was a GE Maxitron tube operating at 250 Kvp, 30 milliamps, at a target distance of 40 cm. The X-rays were filtered through 1 mm. of aluminum. The conidia received approximately 500 r per minute.

Reconstruction Experiments.—To provide an estimation of the mortality rate of mutant conidia during incubation, synthetic mixtures of mutant and wild-type conidia were incubated, filtered, and compared with the irradiated and control suspensions. Each control and irradiation experiment was accompanied by three reconstruction experiments of the following mutant: wild-type ratios: 1:99, 1:999, and 1:9,999. Mortality of the mutant conidia was calculated as per cent conidial death during incubation of the synthetic mixtures. The essential features of this type of experiment have been described.[11]

Calculation of Mutation Rates.—Initial population sizes were estimated by hemocytometer counts. Aliquots of the original population were irradiated, controls excepted, and incubated in minimal medium. Following incubation, the conidial suspension was plated onto minimal agar supplemented specifically to preserve the desired mutant type. Population sizes were again determined, and total conidial mortality was fixed by subtracting the latter total from the original count. (Mutation rates were not based on the initial number of viable conidia as determined by survival on agar medium. It has been shown earlier[12] that low

doses of X-rays initiate germination of conidia that do not germinate without X-irradiation under ordinary conditions of the experiments. It was assumed that higher doses of X-rays would act similarly, but at higher doses the detection of increased germination would be obscured by increased inactivation. On this assumption, the number of conidia that germinate with and without irradiation would be different; therefore, the best measure of the size of conidial populations was the hemocytometer count. It is not known whether the stimulation effects persist throughout incubation, however.) Samples of the population that developed on the supplemented agar were tested to determine the proportion of specific mutants. From the number of mutants isolated, the mutant population of the conidial suspension subsequent to filtration was determined. Then, by extrapolation to the original conidial population, by the use of reconstruction experiment data, the size of the initial mutant population was estimated. The steps of this calculation are illustrated in Table 2.

Mutation rates were calculated on the basis of mutations per conidium; since the strain used was a macroconidial strain, this does not represent the mutation event in terms of mutations per nucleus or chromosome. In addition, the spontaneous mutation rates reported differ from the induced rates, since they represent the accumulation of mutant colonies resulting from clonal effects, as well as those occurring immediately prior to plating.

RESULTS

The mutation rates reported concern only those genes (operational genes) studied whose spontaneous mutation rates were sufficiently high to permit recovery by the filtration method. The results, therefore, are not intended to represent the peculiarities of mutation for the entire gene complex of *Neurospora*.

Table 1 illustrates the results of two reconstruction experiments. Table 2 gives the spontaneous and induced mutation rates for the genes studied. The data of Table 1 were used to calculate columns 10 and 11 of Table 2. For example, the

TABLE 1

ESTIMATION OF MUTANT CONDIAL MORTALITY BY USE OF RECONSTRUCTION EXPERIMENTS

Mutant	Mutant: Wild (Ratio)	Viable Mutant Conidia $\times 10^{-3}$ after Incubation*	Per Cent Survival of Mutant Conidia
Citrulline	1:99	185.0	18.5
	1:999	18.0	18.0
	1:9,999	1.8	18.0
		Av.	18.1
Glutamic acid	1:99	18.5	1.85
	1:999	1.95	1.95
	1:9,999	0.21	2.10
		Av.	2.00

* Each irradiation experiment was accompanied by a set of three reconstruction experiments.

per cent mutant survival during incubation was 18.1 for the citrulline mutant in Table 1. This figure is shown in column 9, Table 2, in the first entry describing the citrulline mutant. It was used to compute the corresponding data of column 10. The remainder of the data in column 10 of Table 2 were similarly calculated.

TABLE 2

SPONTANEOUS AND INDUCED MUTATION RATES OF SEVERAL LOCI OF *Neurospora*

Mutant	Treatment (1)	Population Size (2)	No. Isolates (3)	No. Col. per Plate Comp. Med. (4)	No. Mutants Recovered (5)	*M/T* (6)	No. Conidia Surviving Incubation (7)	No. Mutants Surviving Incubation (8)	Per Cent Mutants Surviving Reenst. (9)	No. Mutants in Initial Population (10)	Mutants 10^6 Con. (11)
Arginine complex	No dose	5×10^7	81	1.7	9	0.11	1,530	170	19.5	872	0.174
	250 r	5×10^7	88	2.1	9	.10	1,890	190	21.0	904	0.181
	10^3 r	4.8×10^7	86	1.95	11	.125	1,655	200	18.2	1,099	0.230
	10^4 r	4.1×10^7	91	2.6	16	.175	2,340	411	18.0	2,283	0.560
	3.5×10^4 r	4.0×10^7	90	3.5	25	.277	3,150	875	17.1	5,116	1.280
	5×10^4 r	7.0×10^7	182	3.8	56	.307	3,420	1,052	16.2	6,500	1.625
Citrulline	No dose	1.5×10^8	243	2.7	9	.037	3,645	135	18.1	747	0.05
	250 r	1.5×10^8	264	2.5	9	.034	3,375	114	17.4	654	0.04
	10^3 r	1.44×10^8	258	2.9	15	.058	3,915	228	18.2	1,252	0.09
	10^4 r	1.23×10^8	273	3.0	26	.095	4,050	385	17.9	2,151	0.18
	2×10^4 r	1.2×10^8	270	2.8	44	.163	3,780	616	18.7	3,294	0.27
	3.5×10^4 r	1.26×10^8	267	2.8	57	.213	3,780	807	18.1	4,458	0.35
	5×10^4 r	1.2×10^8	273	2.5	70	.265	3,375	865	17.5	4,943	0.41
Glutamic acid	No dose	2.07×10^8	243	10.3	7	.029	13,950	396	2.4	16,500	0.8
	250 r	1.07×10^8	94	20.8	2	.021	9,360	200	2.0	10,000	0.93
	10^3 r	1.07×10^8	98	13.0	1	.010	5,850	60	0.5	12,000	1.12
	2×10^3 r	....	..	..	..	..	..	..	..	...	1.03
	3×10^3 r	....	..	..	..	..	..	..	..	...	1.5
	7×10^3 r	....	..	..	..	..	..	..	..	...	3.05
	10^4 r	1.82×10^8	188	12.5	23	.122	11,250	1,376	2.0	68,800	3.78
	3.5×10^4 r	1.67×10^8	163	12.0	40	.245	10,800	,650	1.0	265,000	15.87
	5×10^4 r	7.5×10^8	74	20.0	22	0.297	9,000	2,676	2.0	133,800	17.86

* Col. 1, dose measured with a Victoreen dosimeter; Col. 2, population sizes estimated with a hemocytometer, S.E. 4 per cent; col. 3, colonies from specifically supplemented agar plates placed onto complete agar slants; col. 4, specifically supplemented media; col. 5, initially determined by nutritional test, then verified by heterocaryon and crossing tests; col. 6, number of specific mutants recovered/number of isolates made; col. 7, number of colonies/plate × number of cc. of suspension × $^1/_2$ ($^1/_2$ cc. suspension was placed on each plate); col. 8, calculated as col. 5: col. 3::col. 8:col. 7; col. 9, each figure represents a set of three reconstruction experiments (see Table 1); col. 10, calculated as col. 8 = col. 9 per cent of col. 10; col. 11, calculated as col. 10:col. 2::col. 11:10,000.

More than a tenfold difference exists between the spontaneous mutation rates of the glutamic acid and the citrulline genes. This difference was not evident by the procedural efficacy of mutant recovery of the filtration method but was revealed by the studies of mutant mortality. This finding corroborates earlier predictions[11] concerning the usefulness of the filtration technique by pointing out that the utility of the method depends not only upon the frequency of mutation but also upon mutant viability during incubation.

Attempts were made to alter mutation rates without varying dosage. Stapleton and Hollaender[13] reported that X-rays induced more morphological mutants of *Aspergillus* when the spores were irradiated in a water suspension than when they were irradiated dry. This also was shown to be the case in *Neurospora*.[14] The experiment was repeated, using ultraviolet light (GE germicidal lamp, 8-watt, target distance 5 cm., 15–60-second exposures), and it was shown that the conidia irradiated dry yielded more "colonial" mutations than conidia irradiated in a water suspension (Table 3). Since the difference was nearly fivefold, a wide range

TABLE 3

COMPARISON BETWEEN SPORES EXPOSED TO ULTRAVIOLET LIGHT IN WATER SUSPENSION OR DRY

Condition	No. Isolates	No. Colonial-Type Mutants	Mutants/ No. Isolates
Dry	311	99	0.318
Wet	2,401	157	0.065

of sensitivities no doubt exists, and these seem to warrant the study of varied moisture levels and ultraviolet sensitivity. This study is being continued. Kaplan[15] has shown similar water content–ultraviolet sensitivity relationships to exist in bacteria.

DISCUSSION

Although mutation-rate studies have contributed greatly to genetic theory little has been learned about the chemistry of mutation. All that is really measured is the frequency of phenotypic change. It seems, therefore, premature to attempt the interpretation of phenotypic changes in terms of molecular events at the gene level.

The present study fails to circumvent these general deficiencies; however, in viewing it as a methodological adjunct, the following considerations are put forth:

A. *Mutant Mortality.*—Reconstruction experiments were used as a measure of mutant conidia viability. Two factors that could possibly obscure the true picture are the following: (i) The viability of irradiated conidia may differ from the viability of unirradiated conidia. This should not affect the spontaneous mutation rates, but (ii) mutants of recent occurrence (spontaneous or induced) may show different survival potentials from mutant conidia derived from an established population. This phenomenon was recently described by Witkin and Thomas[16] for bacterial mutants.

B. *Selective Media.*—Lethality must be defined in terms of nutritional environment. A nutritional mutant may be lethal on a "minimal" medium but not on a "complete" medium. Yet many mutants have been recovered that grow well on

minimal medium but are inhibited on complete. Also, a large proportion of the mutants recovered from complete agar cannot be characterized as to nutritional requirement. Since there is no logical definition of lethality except in terms of genotype and nutritional requirement, a selective medium was used that eliminated the ambiguous types and preserved only the desired mutant types and the prototrophs.

C. *Calculation of Mutation Rates.*—The mutation rates were calculated on the basis of the total number of conidia rather than on the basis of total viable conidia. Since most of the conidia that do not germinate on agar medium do germinate after irradiation, it was assumed that the hemocytometer counts more closely revealed the size of the conidial population from which the mutants were derived.

Alterations of mutation "rate" by means other than varying dosage reconfirm the concept that mutation rate is arbitrary, i.e., mutation rates are not constant under all conditions. It is a matter for future research to determine the relations among gene change and the various factors that affect the change.

SUMMARY

The spontaneous and induced mutation rates of several genes governing nutritional requirements in *N. crassa* were estimated. Under the described experimental procedure, the spontaneous mutation rate for the glutamic acid gene was 0.8 per 10^4 conidia and that for the citrulline gene was 0.005 per 10^4 conidia. Mutation rates at both loci increased with increasing X-ray dosage.

The present work does not obviate the shortcomings of past mutation-rate experiments, but it provides a methodology that may prove useful in subsequent studies of gene stability in *Neurospora.*

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SQUARE-INTEGRABLE DIFFERENTIALS ON OPEN RIEMANN SURFACES

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1. In recent years it has been customary to study open Riemann surfaces which share some important property of closed surfaces. Usually this is expressed by the fact that the surface is unable to carry nontrivial harmonic or analytic differentials of some special kind. If one uses square-integrable differentials, it might be expected that results obtained in this manner are capable of generalization in the following direction: instead of imposing restrictions on the surfaces, we may restrict the differentials by orthogonality conditions which become vacuous for surfaces that exhibit a relevant kind of degeneracy.

To carry out this program, it becomes necessary to introduce certain linear subclasses of differentials which have not previously received attention. The purpose of this note is to outline the relations that one is thus led to study.

2. If W is a Riemann surface, we denote by $\Gamma(W)$ or Γ the Hilbert space of square-integrable first-order differentials on W. The subspaces of closed and exact differentials are denoted by Γ_c and Γ_e; these subspaces are to be closed, so that the elementary definition has to be complemented by passing to the closures.

It is convenient to let the differentials in Γ have complex coefficients. Thus $\omega = pdx + qdy$ has the complex conjugate $\bar{\omega} = \bar{p}dx + \bar{q}dy$ and the *adjoint* $\omega^* = -qdx + pdy$. The notations Γ_c^* and Γ_e^* are self-explanatory. $\Gamma_h = \Gamma_c \cap \Gamma_c^*$ is the space of harmonic differentials, and $\phi \in \Gamma_h$ is called analytic if $\phi^* = -i\phi$. Every $\omega \in \Gamma_h$ has a unique representation $\omega = \phi + \bar{\psi}$ through an analytic and an antianalytic differential.

The orthogonal complement of a closed linear subspace $A \subset \Gamma$ is denoted by $A^\perp$. By way of definition, we write $\Gamma_{eo} = \Gamma_c^{*\perp}$ and $\Gamma_{co} = \Gamma_e^{*\perp}$. One shows that $\Gamma_{eo} \subset \Gamma_e \cap \Gamma_{co}$ and $\Gamma_{co} \subset \Gamma_c$. The various orthogonal decompositions $\Gamma = \Gamma_c + \Gamma_{eo}^* = \Gamma_c^* + \Gamma_{eo} = \Gamma_{co} + \Gamma_e^* = \Gamma_{co}^* + \Gamma_e$ can be superimposed and lead to the three-way decomposition $\Gamma = \Gamma_h + \Gamma_{eo} + \Gamma_{eo}^*$, as well as $\Gamma_h = \Gamma_{ho} + \Gamma_{he}^* = \Gamma_{ho}^* + \Gamma_{he}$, where $\Gamma_{ho} = \Gamma_h \cap \Gamma_{co}$, $\Gamma_{he} = \Gamma_h \cap \Gamma_e$.

3. We shall use systematically the letter Ω to denote a Riemann surface of finite connectivity. It is possible to imbed Ω in a larger surface in such a way that

it is bounded by a finite number of analytic contours. Some of the contours may shrink to points, but the pointlike contours can be omitted without loss of generality.

One shows that the class $\Gamma_{ho}(\Omega)$ consists of the differentials in $\Gamma_h(\Omega)$ which vanish along the boundary; it is finite-dimensional. The *Schottky differentials* on Ω are those which have a harmonic extension to the double of Ω. They form the subspace $\Gamma_s(\Omega) = \Gamma_{ho}(\Omega) + \Gamma_{ho}^*(\Omega)$.

Another important subspace is $\Gamma_{ho}(\Omega) \cap \Gamma_{he}(\Omega)$. It is evidently generated by the differentials of *harmonic measures*, i.e., harmonic functions which are 1 on one contour, 0 on the others. To indicate this, we write $\Gamma_{ho}(\Omega) \cap \Gamma_{he}(\Omega) = \Gamma_{hm}(\Omega)$. The orthogonal complement in $\Gamma_h(\Omega)$ is $\Gamma_{hm}^{\perp}(\Omega) = \Gamma_{ho}^*(\Omega) + \Gamma_{he}^*(\Omega)$, where the vector sum is not direct. On the other hand, one sees directly that the orthogonal complement of $\Gamma_{hm}^*(\Omega)$ consists of those differentials which have zero periods along the contours. For lack of a better terminology, such differentials will be called *semiexact*, and we denote the space of semiexact harmonic differentials by $\Gamma_{hse}(\Omega)$.

We obtain $\Gamma_{hse}(\Omega) = \Gamma_{hm}^{*\perp}(\Omega) = \Gamma_{ho}(\Omega) + \Gamma_{he}(\Omega)$. The vector-sum representation can be strengthened to a direct sum: $\Gamma_{hse} = \Gamma_{hm} + (\Gamma_{ho} \cap \Gamma_{hse}^*) + (\Gamma_{he} \cap \Gamma_{hse}^*)$. It should be noted that the second and third terms are not orthogonal.

4. An arbitrary Riemann surface W can be approximated by subregions Ω of finite connectivity. We say that $\sigma_\Omega \in \Gamma(\Omega)$ converges to $\sigma \in \Gamma(W)$ if the Ω-norm $\|\sigma_\Omega - \sigma\|_\Omega$ has the directed limit 0 as Ω approaches W.

By definition, ω is said to be a Schottky differential on W if it is the limit of differentials in the Schottky classes $\Gamma_S(\Omega)$. For the space $\Gamma_S(W)$ one proves without difficulty

THEOREM 1. $\Gamma_S = Cl\,(\Gamma_{ho} + \Gamma_{ho}^*)$, $\Gamma_S^{\perp} = \Gamma_{he} \cap \Gamma_{he}^*$.

In the first statement it is necessary to take the closure of the vector sum $\Gamma_{ho} + \Gamma_{ho}^*$, for although Γ_{ho} and Γ_{ho}^* are closed, it cannot be concluded that $\Gamma_{ho} + \Gamma_{ho}^*$ is closed. We conjecture that there are indeed surfaces for which this vector sum fails to be closed.

As a corollary of the theorem, we conclude that all harmonic differentials on W are Schottky differentials if and only if $\Gamma_{he} \cap \Gamma_{he}^* = 0$, i.e., if there are no single-valued nonconstant analytic functions on W with finite Dirichlet integral (class O_{AD}). This completes a theorem that I proved in 1950.[1]

5. The one-dimensional homology group of W has a subgroup formed by the homology classes of dividing cycles. We may define $\Gamma_{hse}(W)$ as the class of harmonic differentials which have zero periods along the dividing cycles.

It turns out that the orthogonal complement $\Gamma_{hse}^{\perp}$ can be identified with Γ_{hm}^*, provided that we define $\Gamma_{hm}(W)$ as the limit of the classes $\Gamma_{hm}(\Omega) = \Gamma_{he}(\Omega) \cap \Gamma_{ho}(\Omega)$, that is to say, in close analogy with the definition of Schottky differentials on W.

THEOREM 2. *The spaces $\Gamma_{hse}(W)$ and $\Gamma_{hm}^*(W)$ are mutual orthogonal complements relative to $\Gamma_h(W)$.*

It is clear that $\Gamma_{hm}(W) \subset \Gamma_{he}(W) \cap \Gamma_{ho}(W)$, but presumably these classes are in general not identical.

6. To every cycle c on W, or rather to every homology class, there corresponds a unique differential $\omega_c \in \Gamma_{ho}(W)$ with the property that $\int_c \sigma = (\sigma, \omega_c^*)$ for every closed σ. If c is a dividing cycle, then ω_c belongs to $\Gamma_{hm}(W)$. Because of Theorem 2, the following is true:

THEOREM 3. *The differentials ω_c which correspond to arbitrary cycles span $\Gamma_{ho}(W)$, and the ω_c that correspond to dividing cycles span all of $\Gamma_{hm}(W)$.*

The ω_c can be orthogonalized and lead to an orthogonal basis of Γ_{ho} which is adapted to the decomposition $\Gamma_{ho} = \Gamma_{hm} + (\Gamma_{ho} \cap \Gamma_{hse}^*)$.

7. Let $\{A_i, B_i\}$ be a canonical homology basis modulo the dividing cycles. Then Riemann's bilinear relation

$$(\omega, \sigma^*) = \sum_i [\int_{A_i} \bar{\sigma} \int_{B_i} \omega - \int_{B_i} \bar{\sigma} \int_{A_i} \omega]$$

holds rather trivially if, for instance, $\omega \in \Gamma_{ho}$ while σ is semiexact and has only a finite number of nonzero periods.

In order to obtain a more general result, we choose the homology basis in such a way that certain initial sequences $A_1, B_1, \ldots, A_{k(n)}, B_{k(n)}$ are canonical bases for subregions Ω_n which tend to W. We shall refer to the equation

$$(\omega, \sigma^*) = \lim_{n \to \infty} \sum_{i=1}^{k(n)} [\int_{A_i} \bar{\sigma} \int_{B_i} \omega - \int_{B_i} \bar{\sigma} \int_{A_i} \omega]$$

as the *generalized bilinear relation.*

We assume that ω and σ are semiexact. The restriction of ω to Ω can be decomposed according to the following pattern: $\omega = \omega_{1\Omega} + \omega_{2\Omega} + \omega_{3\Omega}$, $\omega_{1\Omega} \in \Gamma_{ho}(\Omega) \cap \Gamma_{hse}^*(\Omega)$, $\omega_{2\Omega} \in \Gamma_{hm}(\Omega)$, $\omega_{3\Omega} \in \Gamma_{he}(\Omega) \cap \Gamma_{hse}^*(\Omega)$. We decompose σ in the same way and find $(\omega, \sigma^*)_\Omega = (\omega_{1\Omega}, \sigma_{1\Omega}^*) + (\omega_{3\Omega}, \sigma_{3\Omega}^*)$. For $\Omega = \Omega_n$ the first inner product can be evaluated by means of the trivial case of Riemann's relation. Hence we are led to the following result:

THEOREM 4. *For any two semiexact differentials $\omega, \sigma \in \Gamma_{hs}$ the validity of the generalized bilinear relation is equivalent to the condition $(\omega_{3\Omega_n}, \sigma_{3\Omega_n}^*) \to 0$.*

The condition is admittedly difficult to analyze, except in very special circumstances.[2] Nevertheless, it brings a certain amount of clarification to a question that is likely to be central in the further development of the theory of open Riemann surfaces.

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ONE-DIMENSIONAL CONTINUOUS CURVES

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The results stated here are proved in two papers submitted to the *Annals of Mathematics*, in one to the *Pacific Journal of Mathematics*, and in one to the *Canadian Journal of Mathematics*. Let $\mathfrak{R}$ be a collection of one-dimensional locally connected compact metric continua such that (1) no two elements of $\mathfrak{R}$ are homeomorphic to

each other and (2) each one-dimensional locally connected compact metric continuum is homeomorphic to some element of $\mathfrak{R}$. Let $\mathfrak{M}$ be the set of those elements of $\mathfrak{R}$ which do not contain any local cut points. Let $\mathfrak{N}$ be the set of those elements of $\mathfrak{M}$ locally imbeddable in the plane or, what is equivalent, those elements each having the property that each open subset of it contains one of the two primitive skew curves.

The principal and key result is

THEOREM I. *Let $M \in \mathfrak{M}$. Then, in order that M be homeomorphic to the universal curve,*[1] *it is necessary and sufficient that M contain no open subset imbeddable in the plane.*

This theorem not only characterizes the universal curve but shows that if an element M_1 of $\mathfrak{M}$ is not the universal curve and is not an element of $\mathfrak{N}$, then it is of a rather mixed type, i.e., M_1 contains two points at which it is of significantly different local structure. In avoiding any local planarity and the existence of local cut points, one is forced (among the elements of $\mathfrak{R}$) to the universal curve.

The next theorem was used in proving Theorem I and is of some independent interest.

THEOREM II. *Let $K \in \mathfrak{R}$. Then, in order that K be the universal curve, it is necessary and sufficient that K be cross-connected.*[2]

The following theorem is a fairly easy consequence of Theorem I.

THEOREM III. *Let $K \in \mathfrak{R}$. Then, in order that K be homogeneous,*[3] *it is necessary and sufficient that K be one of either the universal curve or the simple closed curve.*

This theorem classifies the elements of $\mathfrak{R}$ with respect to homogeneity. In so doing, it settles a question raised by Menger in his book *Kurventheorie* and also in *Fundamenta Mathematicae* (Problem 50) as to whether the simple closed curve was the only homogeneous element of $\mathfrak{R}$.

The next theorem is established by use of a weaker characterization of the universal curve, such also being used in establishing Theorem II (and hence Theorem I).

THEOREM IV. *Let G be any zero-dimensional compact metric group.*[4] *Then G can operate on the universal curve M (as a continuous transformation group) in a fixed-point-free fashion, i.e., so that each element of G other than the identity moves each point of M. Furthermore, the orbit space under the operation may be required to be the universal curve, or, if G is infinite, it may be required to be a regular curve.*

This theorem contributes to questions raised inferentially by Zippin and Smith in their separate articles in the *Proceedings of the Michigan Conference on Topology* (1940). Zippin remarks that in known examples even the dyadic group acts on locally connected continua with a branching process, i.e., not in a fixed-point-free fashion. Smith asks whether a nonperiodic nearly periodic homeomorphism can operate on a connected space of fairly regular local structure. If the universal curve is a space of "fairly regular local structure," then the answer is affirmative. It should also be noted that the theorem asserts the existence of regular almost periodic homeomorphisms operating on the universal curve.

The final theorem given below gives a complete classification of the elements of $\mathfrak{N}$ by means of a rather interesting relationship between $\mathfrak{N}$ and a set $\mathfrak{X}$ of compact 2-manifolds without boundary such that (1) no two elements of $\mathfrak{X}$ are homeomorphic to each other and (2) each compact 2-manifold without boundary is homeomorphic

to an element of $\mathfrak{X}$. To establish the relationship, we need several further definitions.

A collection G of point sets is said to be *diameter-null* if for any $\epsilon > 0$ at most a finite number of elements of G are of diameter greater than ϵ.

If X is a compact 2-manifold without boundary, a continuum M in X is said to be a *frame* of X, provided that (1) $Cl\,(X - M) = X$, (2) the closures of the components of $X - M$ form a diameter-null collection of disjoint 2-cells, and (3) the boundary of each component of $X - M$ is a simple closed curve. Let $K \in \mathfrak{K}$. A *collapsed mapping* of K (if such exists) is a mapping of K whose point inverses are points and simple closed curves in K such that (1) the sum of the nondegenerate point inverses is dense in K and (2) the set of nondegenerate point inverses is diameter-null and is the set of all simple closed curves in K which (individually) do not separate any connected open subset of K. We note that for no $K \in \mathfrak{K}$ does K admit collapsed mappings onto continua not homeomorphic to each other.

THEOREM V. *Let $N \in \mathfrak{N}$. Then there exists a collapsed mapping of N.*

THEOREM VI. *There exists a one-to-one transformation θ of $\mathfrak{X}$ onto $\mathfrak{N}$ such that (1) for $X \in \mathfrak{X}$, $\theta(X)$ is homeomorphic to each frame of X and (2) for $N \in \mathfrak{N}$, $\theta^{-1}(N)$ is homeomorphic to the image of N under any collapsed mapping of N.*

Note that Theorems V and VI assert that all the topological properties of any 2-manifold (of $\mathfrak{X}$) are deducible from properties of the corresponding element of $\mathfrak{N}$.

* This research was supported in part by the National Science Foundation under Grant G1013. Most of it was completed while the author was at the Institute for Advanced Study on leave from the University of Pennsylvania.

[1] The universal curve was originally defined by Menger as a one-dimensional analogue of the Cantor middle-third set. It may be obtained by "punching holes" out of a cube in a particular regular way. Menger showed that every one-dimensional continuous curve could be imbedded in it.

[2] A locally connected continuum X is said to be *weakly cross-connected* if for every two disjoint pairs (A, A') and (B, B') of points of X there exist two disjoint arcs in X containing $A + A'$, and $B + B'$, respectively. A locally connected continuum X is said to be *cross-connected* if for every connected open set D in X for which $Cl\,(D)$ is locally connected, $Cl\,(D)$ is weakly cross-connected.

[3] A continuum X is said to be *homogeneous* if for every sensed pair (A, B) of points of X there is a homeomorphism of X onto itself carrying A onto B.

[4] It has been pointed out to me by Montgomery that no nondegenerate connected compact group can operate effectively on the universal curve and in fact that the group of all homeomorphisms of the universal curve (onto itself) topologized in a natural way contains no nondegenerate connected compact subset.

ON POWER SERIES WITH UNBOUNDED CLUSTER SETS, AND FUNCTIONS OF CLASS H_2 WITH MEAGER SETS OF RADIAL CONTINUITY

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Let K denote the unit circle $|z| = 1$, and D the open unit disk $|z| < 1$ in the complex plane. If the function $f(z)$ is holomorphic in D, and $e^{i\theta} \in K$, we denote the cluster set, and the radial cluster set, of $f(z)$ at $e^{i\theta}$ by $C(f(z), e^{i\theta})$, $C_\rho(f(z), e^{i\theta})$, respectively.

We show that for some classes of holomorphic functions $f(z)$ in D, as a rule (in a sense that involves Baire category) the cluster set of $f(z)$ at every point of K is unbounded (which is equivalent to the assertion that the cluster set contains the point at infinity). This result is then applied to some special classes of functions.

THEOREM. *For every x in some Banach space X, let $f(x, z)$ be a holomorphic function of $z \in D$, and for every $z \in D$, let $f(x, z)$ be a linear functional of $x \in X$. Then it is possible to decompose K into an open set G and its complement $M = K - G$ (a closed subset of K) and to decompose X into an F_σ-set Q of first category and its complement $R = X - Q$ (a residual subset of X) such that, for every $x \in X$, $C(f(x, z), e^{i\theta})$ is bounded for every $e^{i\theta} \in G$, and, for every $x \in R$, $C(f(x, z), e^{i\theta})$ is unbounded for every $e^{i\theta} \in M$.*

If, further, to every $e^{i\theta} \in K$ there corresponds an $x_\theta \in X$ such that $C(f(x_\theta, z), e^{i\theta})$ is unbounded, then, for every $x \in R$, $C(f(x, z), e^{i\theta})$ is unbounded for every $e^{i\theta} \in K$.

Proof:[1] By a *rational arc* we mean an open subarc of K whose end points have principal amplitudes that are rational numbers. Let W be the set of all rational arcs A possessing the following property: for every $x \in X$, $C(f(x, z), e^{i\theta})$ is bounded for every $e^{i\theta} \in A$. We denote by G the union of all the arcs in W, and set $M = K - G$. Then it is obvious that G is an open subset of K and that, for every $x \in X$, $C(f(x, z), e^{i\theta})$ is bounded for every $e^{i\theta} \in G$.

For every natural number n and every rational arc A, let $D_n(A)$ stand for the region

$$1 - n^{-1} < |z| < 1, \qquad z/|z| \in A,$$

and define $E_n(A)$ to be the set of all $x \in X$ such that $|f(x, z)| \leqq n$ for every $z \in D_n(A)$. Then it is evident that

$$E_1(A) \subset E_2(A) \subset \ldots \subset E_n(A) \subset \ldots . \tag{1}$$

For every rational arc A and every natural number n, the set $E_n(A)$ is closed in X. For suppose that $x_k \in E_n(A)$ $(k = 1, 2, 3, \ldots)$ and $\lim_{k\to\infty} x_k = x^*$. Then, for every $z \in D_n(A)$, $|f(x_k, z)| \leqq n$ $(k = 1, 2, 3, \ldots)$, and, since $f(x, z)$ is a linear functional of x, we have $\lim_{k\to\infty} f(x_k, z) = f(x^*, z)$, so that $|f(x^*, z)| \leqq n$, which implies that $x^* \in E_n(A)$.

If A is a rational arc and $A \in W$, then $\bigcup_{n=1}^{\infty} E_n(A)$ is of first category in X. For suppose that ξ is an interior point of $\bigcup_{n=1}^{\infty} E_n(A)$. Then, for every $x \in X$, there exists

a real number $t > 0$ such that $\xi + tx \in \bigcup_{n=1}^{\infty} E_n(A)$. Since $f(x, z)$ is a linear functional of x,

$$f(x, z) = t^{-1}[f(\xi + tx, z) - f(\xi, z)].$$

There exist natural numbers n_1 and n_2 such that $\xi \in E_{n_1}(A)$ and $\xi + tx \in E_{n_2}(A)$, and, if we set $m = \max(n_1, n_2)$, we have, because of formula (1), $\xi \in E_m(A)$ and $\xi + tx \in E_m(A)$. Consequently, for every $z \in D_m(A)$,

$$|f(x, z)| \leqq t^{-1}(|f(\xi + tx, z)| + |f(\xi, z)|) \leqq 2mt^{-1},$$

so that $C(f(x, z), e^{i\theta})$ is bounded for every $e^{i\theta} \in A$, and hence $A \in W$, contrary to assumption. The set $\bigcup_{n=1}^{\infty} E_n(A)$ is thus an F_σ in X containing no interior point, and is therefore of first category in X.

There are only enumerably many rational arcs, so that, if we set

$$Q = \bigcup_{A \in W} \bigcup_{n=1}^{\infty} E_n(A) \qquad (A \text{ denotes a rational arc}), \tag{2}$$

Q is an F_σ of first category, and $R = X - Q$ is a residual subset of X.

Suppose that $x \in R$, $e^{i\theta} \in M$, and $C(f(x, z), e^{i\theta})$ is bounded. Then, for some sufficiently small rational arc A containing $e^{i\theta}$, and some sufficiently large natural number n, $x \in E_n(A)$. Since $x \notin Q$, equation (2) implies that $A \in W$, and hence $e^{i\theta} \in G$, which contradicts our assumption that $e^{i\theta} \in M$. Consequently, for every $x \in R$, $C(f(x, z), e^{i\theta})$ is unbounded for every $e^{i\theta} \in M$.

Now suppose that to every $e^{i\theta} \in K$ there corresponds an $x_\theta \in X$ such that $C(f(x_\theta, z), e^{i\theta})$ is unbounded. Then G is empty, and hence $M = K$, so that, according to the preceding paragraph, for every $x \in R$, $C(f(x, z), e^{i\theta})$ is unbounded for every $e^{i\theta} \in K$.

This completes the proof of the theorem.

Consider the following example. Let $p > 1$,

$$f(x, z) = \sum_{n=0}^{\infty} a_n z^n, \qquad x = \{a_n\}, \qquad a_n \text{ complex} \qquad (n = 0, 1, 2, \ldots),$$

and take X in the above theorem to be the Banach space X_p consisting of all x for which $\sum_{n=0}^{\infty} |a_n|^p < \infty$, where the norm of x is defined to be $||x|| = \left(\sum_{n=0}^{\infty} |a_n|^p\right)^{1/p}$. Denote by S the region in the complex plane that contains the point $2 + {}^1/_5 i$ and whose frontier consists of the point at infinity and the following curves: $\Re(z) \geqq 1$, $\Im(z) = 0$; $\Re(z) = 1$, $0 \leqq \Im(z) \leqq 1$; $\Re(z) \geqq 1$, $\Im(z) = [\Re(z)]^{-2}$. For every $e^{i\theta} \in K$, let $g_\theta(z) = \sum_{n=0}^{\infty} b_n^{(\theta)} z^n$ be a function which maps D in a one-to-one, conformal manner onto S in such a way that the point $e^{i\theta}$ of K corresponds to the point ∞ of the frontier of S. Then $C(g_\theta(z), e^{i\theta}) = \{\infty\}$. The area of S is obviously finite, and hence $\sum_{n=0}^{\infty} n|b_n^{(\theta)}|^2 < \infty$. This implies (with the aid of Hölder's inequality) that $\sum_{n=0}^{\infty} |b_n^{(\theta)}|^p < \infty$. Consequently, if we set $x_\theta = \{b_n^{(\theta)}\}$ $(n = 0,$

1, 2, . . .), we have $x_\theta \in X_p$, $f(x_\theta, z) = g_\theta(z)$, and $C(f(x_\theta, z), e^{i\theta})$ is unbounded. An application of our theorem now yields

COROLLARY 1. *For every $p > 1$, there exists a residual subset R_p of X_p such that, for every $x \in R_p$, $C(f(x, z), e^{i\theta})$ is unbounded for every $e^{i\theta} \in K$, and hence*[2] *$C_\rho(f(x, z), e^{i\theta})$ is unbounded for every $e^{i\theta}$ belonging to some residual subset K_x of K.*

Suppose, in particular, that $p = 2$. Then the class of functions $f(x, z)$ $(x \in X_2)$ is identical with the Hardy class of functions H_2, where the usual norm of $f(x, z)$ coincides with the norm of $x \in X_2$. As is well known, for every function $f(x, z)$ of this class, $\lim_{r \to 1} f(x, re^{i\theta})$ exists and is finite for a set, L_x, of values of θ of Lebesgue measure 2π in the interval $0 \leqq \theta < 2\pi$. If, however, x belongs to the set R_2 of Corollary 1, then, because of the nature of the set K_x of Corollary 1, the corresponding set L_x is only of first category in the interval $0 \leqq \theta < 2\pi$. We state this result as

COROLLARY 2. *There exists a residual subclass of the Hardy class of functions H_2 such that, for every function of this subclass, the set of all radii of K along which this function tends to finite limits (as $|z| \to 1$) intersects K in a set that is only of first category.*

[1] This is a modification of a proof due to C. Ryll-Nardzewski and H. Steinhaus, "Sur les séries de Taylor," *Studia Math.*, **12**, 159–165, 1951.

[2] See E. Collingwood, "Sur le comportement à la frontière, d'une fonction méromorphe dans le cercle-unité," *Compt. rend. Acad. sci.* (Paris), **240**, 1502–1504, 1955.

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*SINGULARITIES OF ANALYTICAL DIFFERENTIAL SYSTEMS IN THE PLANE**

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In this note we consider systems

$$\dot{x} = X = [x, y]_p, \qquad \dot{y} = Y = [x, y]_q, \tag{1}$$

where $[\ ]_r$ designates a power series in x, y convergent near the origin and beginning with terms of degree $\geq r$. The origin is supposed to be an isolated singularity.

A complete classification of the "local phase-portrait" has been accomplished modulo "fans" (the name indicates fairly clearly the nature of the structure). The method followed is essentially geometric.

One may write

$$X = X_p + X_{p+1} + \ldots, \qquad Y = Y_q + Y_{q+1} + \ldots, \tag{2}$$

where X_r, Y_r are forms of degree r. By a preliminary linear transformation one may dispose of the situation so that $p = q$, which is assumed henceforth.

The polynomial $\Delta(x, y) = yX_p - xY_p$ is the *indicator*. When $\Delta \not\equiv 0$ the system is said to be *regular;* otherwise it is said to be *irregular*. As noted already by Bendixson,[1] in the regular case, paths tending to or away from the origin (*TO-*

curves) do so in a finite number of directions. In the irregular case, however, *TO*-curves tend to the origin in every direction. We shall be mainly concerned with the regular case.

Through a combination of the Weierstrass preparation theorem and the Puiseux theorem, one may write the system in the form

$$\dot{x} = \alpha F(x, y) \prod (y - x^{p_i/m_i} E_i(x^{1/m_i}))^{\sigma_i},$$
$$\dot{y} = \beta G(x, y) \prod (y - x^{q_i/n_i} E_j(x^{1/n_j}))^{\tau_j}, \tag{3}$$

where α, β are constants, the factors are real, and F, G are positive near the origin. As a matter of fact, one may even reduce the exponents mod 2 without changing topologically the local phase-portraits, provided that there are no *TO*-curves in the sectors to be deleted.

Let Γ_V, Γ_H denote the real branches of the loci $\dot{x} = 0$, $\dot{y} = 0$. Along a Γ_V branch the tangents to the paths are vertical, and along a Γ_H branch they are horizontal.

Often an important simplification can be made. Let V denote the vector (X, Y), and suppose for the present that we have two consecutive Γ_V branches in the first quadrant. If in the sector between the two branches the vector V points in the second or fourth quadrant, then as regards the topology of the phase-portrait the two branches may be suppressed; similarly regarding Γ_H branches or sectors bounded by one of the two types of branches and one of the axes: the y-axis for the Γ_H branches and the x-axis for the Γ_V branches. On the other hand, when the vector V does not point in the right way (in the above sense), an ambiguity arises and is only to be decided by the application of more weighty methods of the type which borrow from algebraic geometry.

Similar methods involving in one case the use of polar co-ordinates allow one to dispose of the irregular case also.

Applications: Take the system

$$\dot{x} = ax + by + [x, y]_2, \qquad \dot{y} = cx + dy + [x, y]_2.$$

If the characteristic roots of the matrix $\left(\begin{smallmatrix} a & b \\ c & d \end{smallmatrix}\right)$ are $\neq 0$, we have an elementary singularity, and our methods enable one to dispose of them with ease. If one of the characteristic roots is zero, we have a so-called "Bendixson system," and our methods yield his main results, namely, the following local phase-portraits: saddle point, node, half-node and half-saddle-point (two hyperbolic sectors). If both characteristic roots are zero but $\left(\begin{smallmatrix} a & b \\ c & d \end{smallmatrix}\right) \not\equiv 0$, as the author has shown, the system is orthogonal to a Bendixson system. This observation, together with our methods, does yield all the possible types: node, saddle point, focus or center, two hyperbolic sectors (with or without fans), two hyperbolic sectors and a node; a hyperbolic sector adjacent to a nested oval sector.

The possible existence of the combination hyperbolic sector and nested oval sector was pointed out by C. Coleman (thesis, Princeton University, 1955). A searching analysis by Lefschetz showed that this was the only type with a nested oval sector that could arise in the family of differential equations under consideration.

The author wishes to express his appreciation to Professor Solomon Lefschetz for his invaluable direction and encouragement in carrying out the present research.

* A complete exposition of the results outlined here will appear elsewhere.

[1] I. Bendixson, "Sur les courbes définies par des équations différentielles," *Acta Math.*, **24**, 1–88, 1901.

DYNAMIC PROGRAMMING AND LAGRANGE MULTIPLIERS

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1. *Introduction.*—The purpose of this note is to indicate how a suitable combination of the classical method of the Lagrange multiplier and the functional-equation method of the theory of dynamic programming[1] can be used to solve numerically, and treat analytically, a variety of variational problems that cannot readily be treated by either method alone.

A series of applications of the method presented here will appear in further publications.

2. *Functional Equation Approach.*—Consider the problem of maximizing the function

$$F(x_1, x_2, \ldots, x_N) = \sum_{i=1}^{N} g_i(x_i), \tag{2.1}$$

subject to the constraints

$$\begin{aligned} &(a) \quad \sum_{j=1}^{N} a_{ij}(x_j) \leq c_i, \qquad i = 1, 2, \ldots, M, \\ &(b) \quad x_i \geq 0, \end{aligned} \tag{2.2}$$

where the functions $a_{ij}(x)$, $g_i(x)$ are taken to be continuous for $x \geq 0$, and monotone increasing. For $c_i \geq 0$, define the sequence of functions

$$f_N(c_1, c_2, \ldots, c_M) = \operatorname*{Max}_{\{x\}} F(x_1, x_2, \ldots, x_N) \tag{2.3}$$

for $N \geq 1$.

Then $f_1(c_1, c_2, \ldots, c_M)$ is determined immediately, and, employing the principle of optimality,[1] we obtain the recurrence relation

$$f_{k+1}(c_1, c_2, \ldots, c_M) = \operatorname*{Max}_{0 \leq a_{i,\,k+1}(x) \leq c_i} [g_{k+1}(x) + f_k(c_1 - a_{1,\,k+1}(x), \ldots, c_M - a_{M,\,k+1}(x))], \tag{2.4}$$

for $k = 1, 2, \ldots, N - 1$.

Due to the limited memory of present-day digital computers, this method founders on the reef of dimensionality when $M \geq 4$. If we wish to treat applied problems of greater and greater realism, we must develop methods capable of handling problems involving higher dimensions.

In this paper we shall present one method of overcoming these dimensionality difficulties.

3. *Functional Equations and Lagrange Multipliers.*—The method of the Lagrange multiplier in classical variational theory consists of forming the function

$$\phi(x_1, x_2, \ldots, x_N) = \sum_{i=1}^{N} g_i(x_i) - \sum_{i=1}^{M} \lambda_i \left(\sum_{j=1}^{N} a_{ij}(x_j) \right), \tag{3.1}$$

where the λ_i are parameters determined subsequently by means of relations (2.2), and then utilizing a direct variational approach on this new function.

We shall employ an approach intermediate between this method and the method sketched in section 2.

Consider the function

$$F(x_1, x_2, \ldots, x_N; \lambda_1, \lambda_2, \ldots, \lambda_K) = \sum_{i=1}^{N} g_i(x_i) - \sum_{i=1}^{K} \lambda_i \left(\sum_{j=1}^{N} a_{ij}(x_j) \right), \quad (3.2)$$

where $1 \leq K \leq M - 1$. We wish to maximize this function over the region defined by the constraints

$$\begin{aligned} &(a) \quad \sum_{j=1}^{N} a_{ij}(x_j) \leq c_i, \qquad i = K + 1, \ldots, M, \\ &(b) \quad x_i \geq 0. \end{aligned} \quad (3.3)$$

For fixed values of the λ_i, we have a problem of precisely the type discussed in section 2, with the advantage that we now require functions of dimension $M - K$ for a computational solution.

Once the sequence $\{\phi_N(c_{K+1}, \ldots, c_N; \lambda_1, \lambda_2, \ldots, \lambda_K)\}$, $N = 1, 2, \ldots$,

$$\phi_N(c_{K+1}, \ldots, c_N; \lambda_1, \lambda_2, \ldots, \lambda_K) = \operatorname*{Max}_{\{x\}} F(x_1, x_2, \ldots, x_N; \lambda_1, \lambda_2, \ldots, \lambda_K) \quad (3.4)$$

has been computed, we vary the parameters λ_i to determine the range of the parameters $c_1, c_2, \ldots, c_K$.

We have thus partitioned the computation of the original sequence of functions of M variables into the computation of a sequence of functions of K variables, followed by the computation of a sequence of functions of $M - K$ variables. The choice of K will depend upon the process.

There are a number of rigorous details which we will discuss elsewhere.

4. *Successive Approximations.*—Since the Lagrange multipliers are intimately connected with "marginal returns," or "prices," in a number of applied problems, we begin with a certain hold on the process as far as approximate solutions are concerned. Iterative techniques based upon this observation, and the connection with optimal search procedures, will be discussed elsewhere.

5. *Application to the Calculus of Variations.*—In the theory of control processes,[3] one encounters a problem such as that of minimizing a nonanalytic functional such as

$$J_1(u) = \int_0^T |1 - u| \, dt \quad (5.1)$$

over all functions $v(t)$ satisfying the constraints

$$\begin{aligned} &(a) \quad -k_1 \leq v(t) \leq k_2, \qquad 0 \leq t \leq T, \\ &(b) \quad \int_0^T |v(t)| \, dt \leq c_1, \end{aligned} \quad (5.2)$$

where u and v are connected by a relation

$$\frac{du}{dt} = g(u, t, v), \qquad u(0) = a. \quad (5.3)$$

Replacing $J_1(u)$ by

$$J_2(u) = \int_0^T |1 - u| \, dt + \lambda \int_0^T |v(t)| \, dt, \tag{5.4}$$

$\lambda \geq 0$, we can employ functions of two variables in determining the analytic or numerical solution, rather than functions of three variables (cf. an earlier paper[2]). This reduction by one in dimensionality results simultaneously in a tremendous saving in computing time and in a great increase in accuracy of the numerical results.

[1] R. Bellman, "The Theory of Dynamic Programming," *Bull. Am. Math. Soc.*, **60**, 503–516, 1954.

[2] R. Bellman, "Dynamic Programming and a New Formalism in the Calculus of Variations," these PROCEEDINGS, **40**, 231–235, 1954.

[3] R. Bellman, I. Glicksberg, and O. Gross, "On Some Variational Problems Occurring in the Theory of Dynamic Programming," *Rend. Palermo*, Ser. II, **3**, 1–35, 1954.

EIGENFUNCTION EXPANSIONS FOR FORMALLY SELF-ADJOINT PARTIAL DIFFERENTIAL OPERATORS. I

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Let A be a formally self-adjoint differential operator defined on a domain G of Euclidean n-space. In 1953, Gårding and the author,[1] following a general line of argument due to Mautner,[2] independently proved the existence of expansions of the Weyl type in eigenfunctions of $(A - \lambda I)$ for elliptic A, corresponding to every self-adjoint realization of A in $L_2(G)$.[3] An extension to self-adjoint realizations of C^∞-elliptic differential operators with constant coefficients was given by L. Hörmander in 1955.[4] A more general result on expansions in eigenfunctions of $(A - \lambda B)$ for A merely formally self-adjoint and B positive has been announced recently by Gelfand and Kostyucenko,[5] who sketched a proof based upon a theorem of Gelfand on the differentiation and integration of Banach space-valued functions on the line. In their result it is assumed that $B^{-1}A$ has a self-adjoint realization in a special Hilbert space with inner product (Bf, g), and the eigenfunctions obtained are distributions in the sense of L. Schwartz.

It is the purpose of this note to present a proof, using the Gelfand differentiation theorem, of the eigenfunction expansion theorem for A formally self-adjoint and B positive without any assumption on the existence of self-adjoint realizations (which may not exist for A and B with complex coefficients). In the following note we establish existence and regularity of the eigenfunction expansion for $(A - \lambda B)$ with elliptic A under weaker differentiability conditions on the coefficients by use of the Radon-Nikodym theorem. The proofs go over to the case of systems with purely formal alterations.

Let A and B be differential operators with complex coefficients on G of orders s and $2r$, $(,)$ the inner product in $L_2(G)$, $C^j(G)$ the j-times continuously differentiable

functions in G, $C_c^\infty(G)$ the infinitely differentiable functions with compact support in G, $[\phi, \psi] = (B\phi, \psi)$ for $\phi, \psi \in C_c^\infty(G)$.

We assume that (1) the coefficients of A lie in $C^k(G)$, $k = \max\{0, [n/2] + 1 - r\}$; (2) the coefficients of B lie in $C^t(G)$, $t = \max\{r, [n/2] + 1 - r\}$; (3) A is formally self-adjoint, i.e., $(A\phi, \psi) = (\phi, A\psi)$ for $\phi, \psi \in C_c^\infty(G)$; (4) B is positive, i.e., there exists $\zeta \in C^0(G)$ with $\zeta(x) > 0$ for $x \in G$ such that $(B\phi, \phi) \geq (\zeta\phi, \phi)$ for $\phi \in C_c^\infty(G)$.

If λ is a real number, by an eigenfunction of $(A - \lambda B)$ we shall mean a distribution ξ, locally of order $r - [n/2] - 1$, for which $(\xi, A\phi - \lambda B\phi) = 0$ for all ϕ in $C_c^\infty(G)$. By assumption 2, there exists $M \in C^0(G)$, $M(x) > 0$, such that, for $\phi \in C_c^\infty(G)$, $[\phi, \phi] \leq \sum_{|\alpha| \leq r} (MD^\alpha\phi, D^\alpha\phi)$. It may be shown, by transplanting this (r, M)-norm to the unit disk and using known results on the asymptotic eigenvalue distribution for the Dirichlet problem, that there exists $N \in C^0(G)$, $N(x) \geq M(x)$, such that (setting $m = r + [n/2] + 1$), if we let $\|\phi\|^2_{m,N} = \sum_{|\alpha| \leq m} (ND^\alpha\phi, D^\alpha\phi)$ and complete $C_c^\infty(G)$ to a Hilbert space $H_{m,N}$ with respect to this norm, then (a) the elements of $H_{m,N}$ can be identified with a subset of H_B, the completion of $C_c^\infty(G)$ with respect to the inner product $[,]$, which itself may be identified with a subset of the functions square-summable on every compact of G; (b) there exists a sequence $\{f_j\}$ in $H_{m,N}$ and a constant $K > 0$ such that the convex closure of $\{f_j\}$ coincides with the unit sphere in $H_{m,N}$, while $\sum_j \|f_j\|_B = K$. We define the space H_1 as the dual of $H_{m,N}$ with respect to the inner product $[,]$ or, equivalently, as the completion of $C_c^\infty(G)$ with respect to the norm $\|\phi\|_1 = \sup_{\psi \in C_c^\infty(G)} \{[\phi, \psi] \cdot \|\psi\|^{-1}_{m,N}\}$. The elements of H_1 are distributions of order $r - [n/2] - 1$.

THEOREM. *If A and B satisfy conditions 1–4, the eigenfunctions of $(A - \lambda B)$ are complete in H_1 and there exist two sequences $\{\xi_\nu\}$, $\{m_\nu\}$ ($\nu = 1, 2, \ldots$), where each m_ν is a Lebesgue-Stieltjes measure on the line L, ξ_ν is an m_ν-measurable function from L to H_1, $\xi_\nu(\lambda)$ being an eigenfunction of $(A - \lambda B)$ for each ν and λ, having the following properties:*

A. If $\phi \in C_c^\infty(G)$, $c_\nu(\lambda) = [\phi, \xi_\nu(\lambda)]$, then $c_\nu \in L_2(m_\nu)$ and $\|\phi\|^2_B = \sum_\nu \|c_\nu\|^2_{L_2(m_\nu)}$. Thus the mapping of ϕ into $\{c_\nu\}$ may be extended by continuity to an isometric mapping of H_B into $\sum_\nu \oplus L_2(m_\nu)$.

B. If $f \in H_B$, $\{c_\nu\}$ its transform sequence, then $f = \sum_\nu f_\nu$, where $f_\nu = \int c_\nu(\lambda)\, \xi_\nu(\lambda)\, dm_\nu(\lambda)$, the integral converging weakly in H_1 to an element f_ν in H_B.

C. If $\{c_\nu\} \in \sum_\nu \oplus L_2(m_\nu)$, then $\int c_\nu(\lambda)\, \zeta_\nu(\lambda)\, dm_\nu(\lambda)$ converges weakly in H_1 to an element $f_\nu \in H_B$, $\sum_\nu f_\nu$ converges strongly in H_B to an element f for which $\|f\|_B^2 \leq \sum_\nu \|c_\nu\|^2_{L_2(m_\nu)}$. (If T_0 below has a self-adjoint extension in H_B, the sequences $\{\xi_\nu\}$ and $\{m_\nu\}$ may be chosen so that equality holds in property C and H_B is mapped onto $\sum_\nu \oplus L_2(m_\nu)$.)

Proof: By assumption 1, for each $\phi \in C_c^\infty(G)$ there exists $k(\phi) > 0$ such that for all $\psi \in C_c^\infty(G)$, $|(A\phi, \psi)| \leq k(\phi)(\zeta\psi, \psi) \leq k(\phi)\|\psi\|_B$. There exists a uniquely defined element $T_0\phi \in H_B$ for which $[T_0\phi, \psi] = (A\phi, \psi)$ for $\psi \in C_c^\infty(G)$. T_0 considered as an operator in H_B, with domain $C_c^\infty(G)$ dense in H_B, is symmetric. We

shall obtain an eigenfunction expansion corresponding to each of the nonempty set of generalized spectral resolutions of T_0 in the sense of Neumark,[6] i.e., the projections upon H_B of the spectral family $\{E_\lambda\}$ of a self-adjoint extension T^+ of T_0 in a separable Hilbert space H^+ containing H_B as a closed subspace. (For example, $H^+ = H_B + H_B'$, H_B' a copy of H_B, then the transformation T' with domain $C_c^\infty(G) \oplus C_c^\infty(G)'$, equal to T_0 on the first component, $(-T_0)$ on the second, will have equal defect indices.)

Choose a complete generating sequence $\{g_v\}$ $(v = 1, 2, \ldots)$ for H^+ with respect to the spectral family $\{E_\lambda\}$, i.e., such that the space H_v^+ spanned by $\{E_S g_v, S$ Borel set of $L\}$ are mutually orthogonal and span H^+. Let $m_v(S) = [E_S g_v, g_v]$. If $f \in H^+$, f_v^+ its projection on H_v^+, there exists $c_v \in L_2(m_v)$ with $\|f_v^+\|_B = \|c_v\|_{L_2(m_v)}$, for which $f_v^+ = \int c_v(\lambda)\, dE_\lambda g_v$.

Let P^+ be the projection op. of H^+ onto H_B, $f_v = P^+ f_v^+$. If $f \in H_B$, $f = \sum f_v$, while $f_v = \int c_v(\lambda)\, dP^+ E_\lambda g_v$. For a fixed v, consider $h(\lambda) = P^+ E_\lambda g_v$. If $f_0 \in H_B$, $\{\Delta_i\}$ a sequence of disjoint intervals of L, $h(\Delta)$ the interval function obtained from $h(\lambda)$, then $\sum_i |[h(\Delta_i), f_0]| \leq \sum_i \|E_{\Delta_i} g_v\| \cdot \|E_{\Delta_i} f_0\| \leq \|g_v\|\, \|f_0\|$. For fixed f_0 in H_B, $[h(\lambda), f_0]$ is absolutely continuous with respect to m_v, for if $\sum_i m_v(\Delta_i) < \delta^2$, then $\sum_i |[h(\Delta_i), f_0]| \leq \sum_i \|E_{\Delta_i} g_v\|\, \|E_{\Delta_i} f_0\| \leq (1/2\delta) \sum_i \|E_{\Delta_i} g_v\|^2 + (\delta/2) \sum_i \|E_{\Delta_i} f_0\|^2 \leq (\delta/2)\,(1 + \|f_0\|_B^2)$.

Let $\{f_j\}$ be the sequence from $H_{m,N}$ described in result (b), $\{u_j^{(i)}\}$ any finite double sequence of real numbers, $0 \leq u_j^{(i)} \leq 1$, and let $f^{(i)} = \sum_j u_j^{(i)} f_j$. The various elements $f^{(i)}$ range independently over a set dense in the convex closure of $\{f_j\}$, which, by result (b), is the unit sphere in $H_{m,N}$. However, $\sum_i |[h(\Delta_i), f^{(i)}] = \sum_{i,j} |u_j^{(i)}| \cdot |[h(\Delta_i), f_j]| \leq \|g_v\| \cdot \sum_j \|f_j\|_B \leq KV$. Thus $\sum_i \|h(\Delta_i)\|_{H_1} \leq KV$. This last condition, together with the weak m_v absolute continuity of h, is sufficient by a theorem of Gelfand[7] to insure that h as a function from L to H_1 has a strong derivative $\xi_v(\lambda)$ with respect to m_v, such that, for $f \in H_{m,N}$, $[h(\lambda), f] = \int_{-\infty}^{\lambda} [\xi_v(u), f]\, dm_v(u)$. As a consequence, for $g \in H_{m,N}$, $[f, g] = \sum_v \int c_v(\lambda)\, [\xi_v(\lambda), g]\, dm_v(\lambda)$ for $f \in H^+$, while, by the spectral theory, $[f, g] = \sum_v \int c_v'(\lambda)\, \overline{c_v(\lambda)}\, dm_v(\lambda)$, where c_v' and c_v are the elements of $L_2(m_v)$ corresponding to f_v^+, g_v^+. Since $\{c_v^{+\prime}\}$ fill out $\sum_v \oplus L_2(m_v)$, $c_v(\lambda) = [g, \xi_v(\lambda)]$ except for a set of m_v-measurable zero. Similarly, for $\phi \in C_c^\infty(G)$, $[T\phi, \xi_v(\lambda)] = \lambda[\phi, \xi_v(\lambda)]$. By continuity this implies $(A\phi, \xi_v(\lambda)) = \lambda(B\phi, \xi_v(\lambda))$, i.e., $\xi_v(\lambda)$ is an eigenfunction of $(A - \lambda B)$.

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ON THE IMBEDDING OF A FINITE COMMUTATIVE SEMIGROUP OF IDEMPOTENTS IN A UNIQUELY FACTORABLE SEMIGROUP*

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A semigroup is a set of elements to which is related an operation usually called *multiplication* and an equivalence relation, such that the set is closed and associative relative to the operation.

We shall discuss, briefly, finite semigroups which are uniquely factorable in the same sense as the multiplicative semigroup of all nonzero integers. Clifford[1] defined *an arithmetic* in such a way as to include our uniquely factorable semigroups as well as similar infinite commutative semigroups, not necessarily cancellable, and gave necessary and sufficient conditions for a commutative semigroup to be imbedded in an arithmetic. He used ideals to accomplish this imbedding. We shall accomplish a constructive imbedding of any finite commutative semigroup of idempotents alone in a uniquely factorable semigroup, by the use of correspondences, or generalized substitutions. This method of imbedding is the chief novelty herein.

Let a finite commutative semigroup S contain an identity, and U be the set of units, or divisors of the identity. Then U is a group, and S can be divided exhaustively into disjoint cosets[2] relative to U. This division into cosets is unique, and the cosets form a semigroup, designated by S/U, whose identity is U, such that S/U is a homomorphic image of S. We shall use $=$ and $\approx$ as the equivalence relations of S/U and S, respectively. If $s \in S$, the elements of the coset sU are called associated elements or associates.

If S is a finite semigroup, $a \in S$, and k and s are the least positive integers such that $k < s$ and $a^k \approx a^s$, then $s - 1$, k, and $s - k$ are called, respectively, the *order*, *index*, and *period* of a.

The semigroup S is called a *uniquely factorable semigroup*, abbreviated "UFS," provided that S is finite and commutative and contains at least two elements, one of which is a unit, and satisfies:

(i) There exists an element in S/U, called a *prime*, and denoted by P, such that the only divisors of P are U and P.
(ii) Each element of S/U has unity for its period.
(iii) Each element of S/U is expressible uniquely, except for order, as a product of powers of distinct primes.

Note that if P^i and P^j are the highest powers of the prime P which appear, respectively, in two factorizations of an element of S/U, it follows that $P^i = P^j$ but not necessarily that $i = j$.

Properties of Uniquely Factorable Semigroups.—The first five of the properties below follow from the fact that S/U is a homomorphic image of S.

1. If S is a UFS, so also is S/U.

2. If S is a UFS and $p \in S$, then p is prime in S (has no other divisors than units and its associates) if and only if the coset pU is prime in S/U.

3. Let S be a UFS, i be an idempotent of S, and G be the maximal subgroup of S with i as its identity; then $G = iU$.

4. If S is a UFS, $s \in S$, $s \in U$, s has index j, and sU has index k, then $j = k$.

5. If S is a UFS, $s \in S$, $s \notin U$, and two complete factor decompositions of s in S are described by $s \approx u_1\left(\prod_{n=1}^{a}(p_n)^{i_n}\right) \approx u_2\left(\prod_{n=1}^{b}(q_n)^{j_n}\right)$, with the i's and j's equal to or less than the indices of the p's and the q's, respectively, and $u_1, u_2 \in U$, then the q's are associates of the p's in some order, $a = b$, and the corresponding exponents are equal.

6. If S is a UFS, $P_1, P_2, \ldots, P_n$ are the nonequivalent primes of S/U, and if these primes generate, respectively, the cyclic groups $S_1, S_2, \ldots, S_n$ and $+$ denotes set union, then S/U is isomorphic to the direct product $(S_1 + U) \times (S_2 + U) \times \ldots \times (S_n + U)$.

Proof: Let the orders of $P_1, P_2, \ldots, P_n$ be $o_1, o_2, \ldots, o_n$, and consider the distinct elements, for $i = 1, 2, \ldots, n$ of the sets $U, \pi_1, \pi_2, \ldots, \pi_n$, where π_1 is the set $P_i, P_i^2, \ldots, P_i^{o_i}$, and $\pi_j, j = 2, 3, \ldots, n$ denotes the set of all products of all the powers of the distinct primes of π_1, taken j at a time. No two elements contained in any of the sets above are equal; otherwise condition (iii) would be violated; and the totality of these elements are the distinct elements of the semigroup $S = (S_1 + U)(S_2 + U)\ldots(S_n + U)$, which is isomorphic to the direct product in the statement of the theorem, since U is the intersection of each pair of parenthesized semigroups.

7. If S is a UFS, each element of which is an idempotent, then S is expressible as a direct product $(p_1 + e) \times (p_2 + e) \times \ldots \times (p_n + e)$, where the p's are the prime idempotents of S and e is the identity of S.

This property is an immediate consequence of property 6.

8. If S_i is a semigroup of order 2 with zero, 0_i, and identity, e_i, for $i = 1, 2, \ldots, n$, then $S = S_1 \times S_2 \times \ldots \times S_n$ is a UFS.

Proof: S is commutative, since each S_i is commutative. Condition (i) is satisfied, since the irreducible elements, or primes, $P_i = (e_1, e_2, \ldots, e_{i-1}, e_i, e_{i+1}, \ldots, e_n)$ generate S. Condition (ii) is satisfied, since each idempotent has period 1, and since S is isomorphic to S/U. Condition (iii) is satisfied by definition of direct product.

9. If S is a UFS and $C \in S/U$, and $C = \prod_{n=1}^{t}(P_n)^{i_n}$ is a factorization of C into powers of distinct primes, then the index of C is the index of the $(P_n)^{i_n}$ with greatest index.

Proof. By condition (ii) the period of C is 1. Let the index of the $(p_n)^{i_n}$ with greatest index be j, and the index of C be k. Then by condition (ii), $\prod_{n=1}^{t}(P_n)^{i_n(j+1)} = \prod_{n=1}^{t}(P_n)^{i_n j} = C^{j+1} = C^j$. Hence $j \geqq k$. But $C^{k+1} = C^k = \prod_{n=1}^{t}(P_n)^{i_n(k+1)} = \prod_{n=1}^{t}(P_n)^{i_n k}$. It follows from condition (iii) that $i_n k$ and $i_n j$ are each greater than or equal to the index of P_n; and, by the definition of the index of $(P_n)^{i_n}$, $k \geqq j$. But $j \geqq k$; therefore, $j = k$.

Correspondences.—C is said to be a correspondence on the set N, provided that

N is finite and C maps each element a of the set N onto some element b of the set N. This is described by $C(a) = b$. We think of a substitution as being a particular correspondence. If we use the double-lined notation of substitution group theory, a correspondence may have repeated elements in the second line. We define equivalence, $\cong$, of correspondences by $C_1 \cong C_2$ if and only if $C_1(a) = C_2(a)$ for each $a \in N$, and the product C_1C_2 of correspondences by $C_1C_2(a) = C_2(C_1(a))$ for each $a \in N$. It follows that each closed set of correspondences is a semigroup. Vandiver and the writer[3] gave examples of the above and other definitions for correspondences and stated a number of results about them.

Let S be a semigroup of correspondences on a set N and n, $e \in N$, such that if $C \in S$, then $C(e) = e$, and, further, $C(n) = e$ implies that n is e; then e is called *extraneous* relative to S.

LEMMA 1. *If S is a semigroup of correspondences on N and e is extraneous relative to S, then S is isomorphic to a semigroup S' on the set N' obtained by deleting e from N.*

Outline of proof: Equivalence in S is independent of e. If for each $C \in S$, and $n \in N'$, we define C' by $C(n) = C'(n)$, then it follows that the (C')'s form a semigroup S' which is isomorphic to S.

If a semigroup of correspondences S operates on N and no element of N is extraneous relative to S, then N is called a *reduced set* for S.

LEMMA 2. *For each commutative semigroup S composed only of idempotent correspondences on a set N, there exist an isomorphism H and $b \in N$ such that if $C \in S$, $H(C) = C'$, and $a \in N$ for which $C'(b) = b$, and whenever $C(a) \neq a$, then $C'(a) = b$, but if $C(a) = a$, then $C'(a) = a$.*

Outline of proof: For each $C \in S$, we define C' such that if $a \in N$ and $C(a) = a$, then $C'(a) = a$, and such that if $d \in N$ and $C(d) \neq d$, then $C'(d) = b$, where $b \in N$ such that for each $C \in S$, $C(b) = b$. The element of N which the zero element of S maps onto itself satisfies this description of b. We consider the three cases $C_1(a) = C_2(a) = a$, $C_1(a) = a \neq C_2(a) = d$, and $C_1(a) = d \neq a$, $C_2(a) = e \neq a$, from which case it follows that $C_1C_2(a) \neq a$. We find in each case that $C_1'C_2'(a) = (C_1C_2)'(a)$. It follows that the set S' of (c')'s is a semigroup and that if H is a mapping such that $H(C) = C'$, then H is an isomorphism.

THEOREM. *Each commutative semigroup S each element of which is an idempotent can be imbedded in a UFS.*

Proof: If S contains only one idempotent, we adjoin a zero element to obtain the desired UFS. It is known[4] that each finite semigroup can be imbedded in a semigroup of correspondences. The proof is constructive and similar to the usual proof of Cayley's theorem. Hence, by this theorem and Lemmas 1 and 2, each finite commutative semigroup S of idempotents alone can be imbedded in a semigroup S' on a reduced set N for S' such that there exists $m_0 \in N$ for which $C'(m_0) = m_0$, and, if $C' \in S'$, $a \in N$, and $C'(a) \neq a$, then $C'(a) = m_0$. We denote the elements of N by $m_0, m_1, \ldots, m_n$ and consider the correspondences $E, P_1, \ldots, P_n$, defined by $P_i(m_i) = m_0$, $P_i(m_j) = m_j$ for $j = 0, 1, \ldots, n$, except $j = i$, and for $i = 1, 2, \ldots, n$, and $E(m_i) = m_i$ for $i = 0, 1, \ldots, n$. Each of these P's is a nonidentity idempotent. Consider the semigroups S_i, $i = 1, 2, \ldots, n$, such that S_i is of order 2 and contains E and P_i. Since each S_i satisfies the hypothesis of property 8 for UFS's, it follows that the semigroup $S'' = \prod_{i=1}^{n} S_i$, which is isomorphic to the

direct product of the S_i's, is a UFS. S'' contains S', since for $C' \in S'$, such that $C' \cong \begin{pmatrix} m_1\, m_2 & & m_r\, m_0 \\ & \cdots & \\ m_0\, m_0 & & m_0\, m_0 \end{pmatrix}$, $r \leqq n$, we may select a set $Q_1, Q_2, \ldots, Q_r$ of elements of the set of P's such that for $i = 1, 2, \ldots, r$, $Q_i(m_i) = m_0$, $Q_i(m_0) = m_0$, $\prod_{i=1}^{\Omega} Q_i \cong C'$. Hence S'' contains a semigroup which is isomorphic to S, and S is imbedded in S''.

Example: Imbed the semigroup S, whose multiplication table appears below, in a UFS.

	0	a	b	c
0	0	0	0	0
a	0	a	b	0
b	0	b	b	0
c	0	0	0	c

The semigroups S' and S'', described in the proof of the theorem, have elements described by

S': $\begin{pmatrix} 0 & a & b & c \\ 0 & 0 & 0 & 0 \end{pmatrix}$, $\underline{\begin{pmatrix} 0 & a & b & c \\ 0 & a & b & 0 \end{pmatrix}}$, $\begin{pmatrix} 0 & a & b & c \\ 0 & 0 & b & 0 \end{pmatrix}$, $\begin{pmatrix} 0 & a & b & c \\ 0 & 0 & 0 & c \end{pmatrix}$;

S'': the union of S' with the elements

$$\underline{\begin{pmatrix} 0 & a & b & c \\ 0 & 0 & b & c \end{pmatrix}}, \quad \underline{\begin{pmatrix} 0 & a & b & c \\ 0 & a & 0 & c \end{pmatrix}}, \quad \begin{pmatrix} 0 & a & b & c \\ 0 & a & 0 & 0 \end{pmatrix}, \quad \begin{pmatrix} 0 & a & b & c \\ 0 & a & b & c \end{pmatrix}.$$

The constructive proof mentioned in footnote 4, together with Lemma 2, is useful in constructing S'. The three underlined elements above are the P's mentioned in the proof. They are also the primes of S''.

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PARTIAL FOURIER SYNTHESES AND THEIR APPLICATION TO THE SOLUTION OF CERTAIN CRYSTAL STRUCTURES

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Introduction.—In an earlier contribution[1] attention was directed to a type of crystal which could be said to have a substructure. Such a crystal has a relatively complex pattern of atoms, yet the pattern is somehow based upon a complication of a simpler pattern. The simpler pattern is called the "substructure," while the set of atoms responsible for the complication is called the "complement structure."

In a simple example, the substructure is a pattern of atoms having a small unit cell, and the complement structure comprises an additional atom added to it periodically in such a way as to create a superstructure having a unit cell, one or more of whose edges are multiples of the edges of the cell of the substructure. A multiple cell can arise not only by addition of atoms to a simpler structure but obviously also by omission of atoms from a simpler structure or by substitution of atoms in a simpler structure. Evidently, it can also arise as a combination of any two or more of these situations.

Definitions.—From one point of view, the superstructure is a derivative structure.[2] This identification is not stressed, since the substructure and complement-structure aspects are required here for formal purposes of aiding in the solution of crystal structures. From this restricted point of view, the substructure is defined as comprising that part of the electron density which conforms to the substructure periods, while the complement structure comprises that portion which does not. This permits one to bring into substructure theory a distortion of a simple structure. In this case, that portion of the electron density which still occupies the electron density of the simple structure pertains to the substructure, while that portion of the electron density which has been distorted out of the substructure periodicity pertains to the complement structure.

Separation of the Complement Structure.—At least one of the identity periods of the superstructure is a multiple of the corresponding period of the substructure. It follows that the set of X-ray diffraction spectra of the superstructure contains a set of superstructure reflections as compared with the set of reflections to be expected from the substructure alone. The complement structure alone is responsible for these superstructure reflections.

To see how this situation may be useful, consider the Fourier transformation of the superstructure. Since Fourier transforms are additive, the transform of the superstructure is the sum of the transforms of the substructure and of the complement structure. The transform of the substructure is zero everywhere except at the reciprocal lattice points which correspond to the sublattice of the substructure. The transform of the complement structure is zero everywhere except at the reciprocal lattice points which correspond to the lattice of the complement structure, which is identical with the lattice of the superstructure. There are n times as many reciprocal lattice points reciprocal to the complement structure

as there are reciprocal to the substructure, where n is the volume multiplicity of the unit cells of the complement structure with respect to the substructure. A simple example, where $n = 2$, is shown in Figure 1. Let the additional reciprocal lattice points present in the reciprocal lattice of the complement structure and not in the substructure be called "excess points."

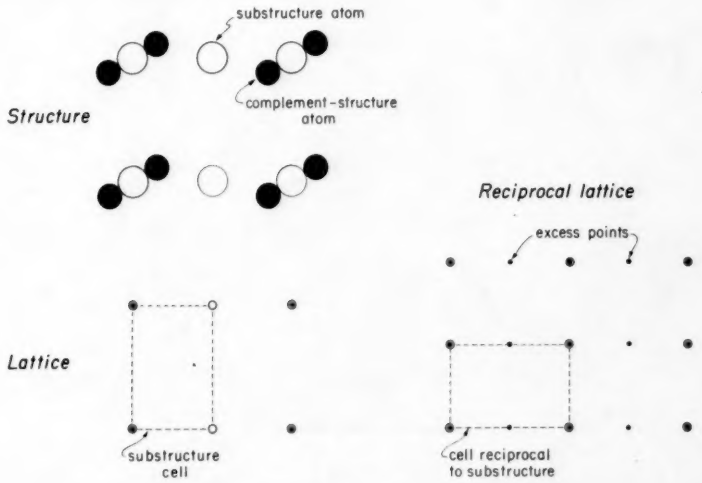

FIG. 1

The Fourier transform of the complement structure is contained both in the excess points and in the substructure points (Fig. 1). The excess points contain contributions from the complement structure only. Inversely, the Fourier transform of the excess points plus the Fourier transform of that portion of the weighting of the substructure points due to the complement structure is the complement structure itself. The question arises, what is the Fourier transform of the excess points alone? It will be shown that it is simply a pattern of the scaled-down electron density of the complement structure.

To see this, consider the Fourier transform of a single unit cell of the complement structure. This transform is a continuously varying function. The Fourier transform of the repetition of these cells on a lattice is a periodic sampling of the Fourier transform of the unit cell, the sampling occurring at the points of the reciprocal lattice of the complement structure. If the sampling interval is arbitrarily changed, the result is the same except for scale of weighting.

Thus the complement structure is separately accessible from a study of the excess points of the reciprocal lattice. If the proportion of excess points to total points is $(n - 1)/n$, then the Fourier transform of the excess points provides the electron density of the substructure on a weighting scale $(n - 1)/n$ times that which would be obtained if all the points could be used. The Fourier transformation is ordinarily found by Fourier summation in which the weights of the reciprocal lattice points are Fourier coefficients. If a substantially infinite set of coefficients

is not available, the use of only the excess points may lead to a slight distortion of the electron density as compared to the electron density found by using all points.

Electron-Density Synthesis of the Complement Structure Alone.—If the phases of the excess points are known, the electron density of the complement structure can

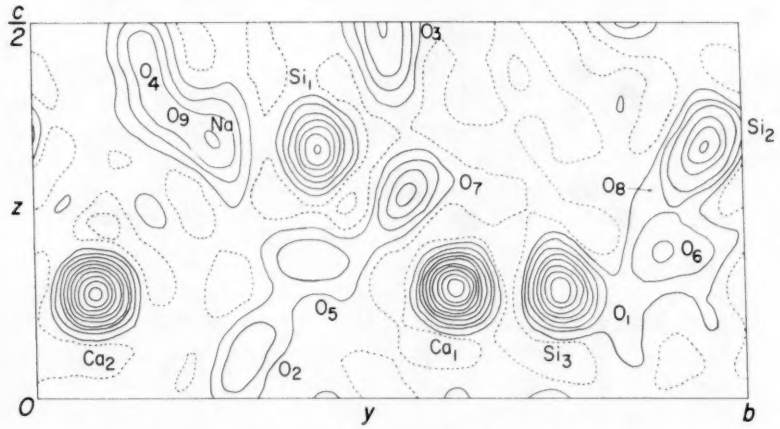

FIG. 2.—Pectolite, $\rho(yz)$

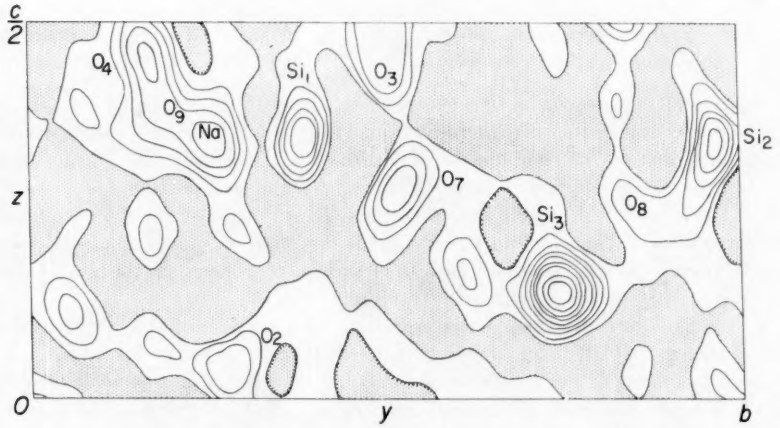

FIG. 3.—Pectolite, $\partial\rho(yz)$. Note vanishing of Ca_1, Ca_2, O_5, and O_6, which are in the substructure.

be found by Fourier synthesis. This can be useful in the event that the atoms of the substructure and the atoms of the complement structure are unresolved in projection. In this event the electron density of the complement structure can be found by Fourier synthesis of the properly phased excess points. An example of synthesis of a complement structure is shown in Figures 2 and 3.

For Figure 3 the Fourier summation has the form

$$\sum_{k \text{ odd}} F_{kl} \cos 2\pi(ky + lz).$$

Since only odd-order terms in k are used, the map at any set of co-ordinate values yz is the negative equivalent of that at $y + \frac{1}{2}$, z. The map therefore displays the plane symmetry $p2b^-$, which is one of the antisymmetries permitted to two-

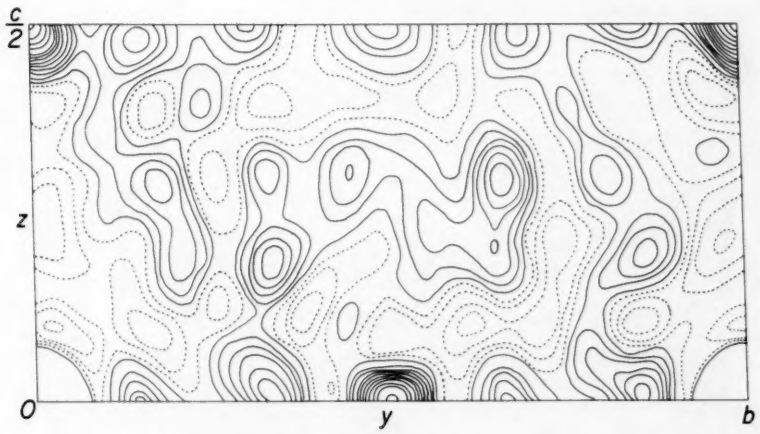

FIG. 4.—Pectolite, $P(yz)$

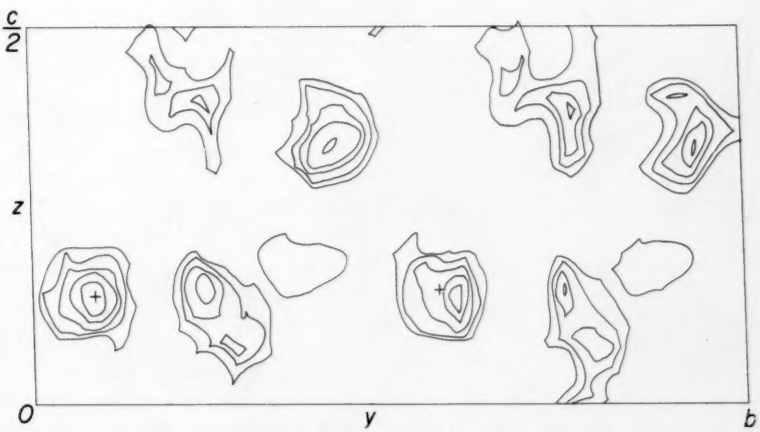

FIG. 5.—Pectolite, $M_2(yz)$

dimensional real periodic functions.[4] Equivalent positive and negative peaks bear an interesting relation to one another. Both are alternative regions permitted to the corresponding complement-structure atom by the substructure. The place where the atom actually *is*, is indicated by the positive peak, while the place where it actually *is not*, is indicated by the negative peak. To emphasize

the places where complement-structure atoms actually are, the negative regions of the maps in Figures 3 and 5 are shaded but not contoured.

Patterson Synthesis of the Complement Structure Alone.—In the event that the phases of the excess points are unknown, which is usually the case, the weights of the phaseless points can be used in Fourier synthesis to form a Patterson function. This has proved to be a most useful application and has already aided in the investigation of several crystal structures.

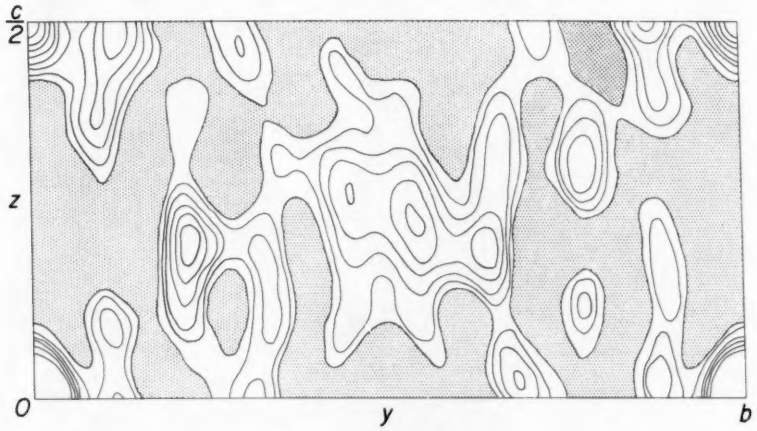

FIG. 6.—Pectolite, $\partial P(yz)$

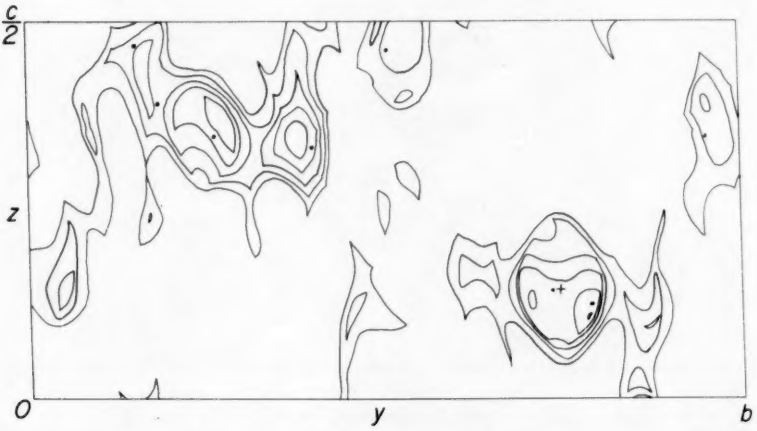

FIG. 7.—Pectolite, $\partial M_2(yz)$

To illustrate the use of such Pattersons, the Patterson map $P(yz)$ of the structure of pectolite, $Ca_2NaHSi_3O_9$, is shown in Figure 4. This shows a strong quasi-periodicity of $b/2$, indicating a substructure having this half-period.[1] The minimum function,[3] $M_2(yz)$, based upon Ca-Ca rotation vectors as line images, is shown

in Figure 5. This map is so dominated by the dense substructure that the important complement structure is obscured, and, furthermore, it is doubled because the structure provides two equal Ca-Ca rotation vectors per cell. The partial Patterson, $\partial P(yz)$, of the substructure alone is shown in Figure 6. Its solution by a minimum function $\partial M_2(yz)$ of the partial Patterson is shown in Figure 7. The dots show the atom locations found by Fourier synthesis (Fig. 2).

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*A NEW THEORY OF LIMB INDUCTION**

BY B. I. BALINSKY

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In 1925 I reported that transplantation of an ear vesicle into the flank of amphibian (*Triton*) embryos may lead to the induction of a supernumerary limb.[1] The experiments were repeated and corroborated by several investigators, but very little was done to find out in what way the grafted inductor (ear vesicle or nose rudiment) causes the formation of a supernumerary limb bud. The nearest approach to this problem was made in my own experiments,[2] in which I found that the induction is accompanied by an increase in the mitotic index in the mesodermal cells participating in limb-bud formation. I interpreted this to mean that a higher level of metabolic activity brought about by the influence of the inductor allows the cells of the body wall to go through a more complex morphogenetic process (limb development), which they are not able to perform while their over-all physiological activity is low, as is normally the case in the flank area.

A method is now available to estimate the metabolic turnover of cells directly; this is the radioactive tracer method. Accordingly, I have performed experiments to test the incorporation of tagged protein and nucleic acid precursors into cells undergoing limb induction. In connection with the views proposed by Brachet,[3] I have also checked the comportment of nucleic acids in limb induction. The standard operation for limb induction—transplantation of the olfactory rudiment into the flank—was performed on *Amblystoma punctatum* embryos in the late tail-bud stage (Harrison's stage 34). At the time when the induced limb buds appeared (in stages 42–44), either the embryos were preserved for staining in sections with Azur B, or solutions of C^{14}-phenylalanine or C^{14}-adenine were injected directly into the gut. The injected larvae were preserved after 3, 6, or 24 hours, sectioned, and radioautographs were prepared, using the Ilford G-5 Nuclear Track Emulsion.

Pretreatment of sections with RNase or DNase was used to differentiate between the uptake of the C^{14}-adenine into the DNA and RNA, respectively.

The results of these experiments are as follows. The concentration of cytoplasmic RNA in mesodermal cells of the induced limb rudiments was found to be about double that in the nonlimb mesoderm cells of the body wall. The uptake of C^{14}-phenylalanine into the cells of an induced limb rudiment is not appreciably different from the uptake into mesenchyme cells of the body wall. The uptake of C^{14}-adenine into the RNA of induced limb-bud cells is about double, and the uptake of the same substance into the DNA of corresponding cells is about three times as high as the uptake into the mesenchyme cells of the body wall. As a certain increase in uptake of both protein and nucleic acid precursors would be expected, since the limb bud is a growing structure, I find that the information obtained does not justify the conclusion that the appearance of a qualitatively new formation (the supernumerary limb) can be satisfactorily accounted for as the result of a local stimulation of metabolic turnover.

Having thus failed to justify my previous expectations, I have attempted to investigate whether there are any peculiarities in the structural relationships between cells involved in limb induction (the cells of the nose rudiment, the mesenchyme, and the epidermis) which could account for their behavior. For this purpose I prepared electron micrographs of parts of the flank of operated larvae. A study of the micrographs at once showed me that there is a very marked difference in the relation to the epidermis in mesenchyme cells of the body wall and in the mesenchyme cells of induced limb rudiments. In the normal skin, at the time when the induced limb buds appear, the epidermal layer possesses on its inner surface a well-developed basement membrane consisting of several layers of fibers crossing each other at right angles, as has already been described[4] (Fig. 1). In the limb bud the basement membrane is completely absent (Fig. 2). The mesenchyme cells are partially separated from the epithelial cells by narrow intercellular spaces, which, however, do not contain an organized fibrous layer. At other points the mesenchyme cells come into direct contact with the inner surface of epithelial cells. The absence of the basement membrane in the area of limb induction may be noted in preparations stained with Mallory-Azan connective-tissue stain, though without the electron micrographs the interpretation of the latter preparations cannot be made with the necessary degree of certainty.

The above information makes it possible for me to propose an entirely new theory of limb induction. It may be taken as being firmly established that limb development can proceed only when there is a harmonious interaction between the mesoderm and epithelium of a limb rudiment.[5, 6] In this interaction the mesoderm not only causes the epithelium to react but is itself controlled by an influence exerted by the epidermis. Stimuli of an inductive nature thus have to pass both ways between the mesoderm and the epithelium. The transmission of an inducing stimulus is in some cases at least (neural-plate induction, lens induction) dependent on a direct contact between the acting and the reacting components. If the same were true in the case of a limb rudiment, one would expect that the intervention of a well-developed fibrous basement membrane would effectively preclude the possibility of limb development even if the components (mesenchyme and epidermis) were capable of participating in such a process.

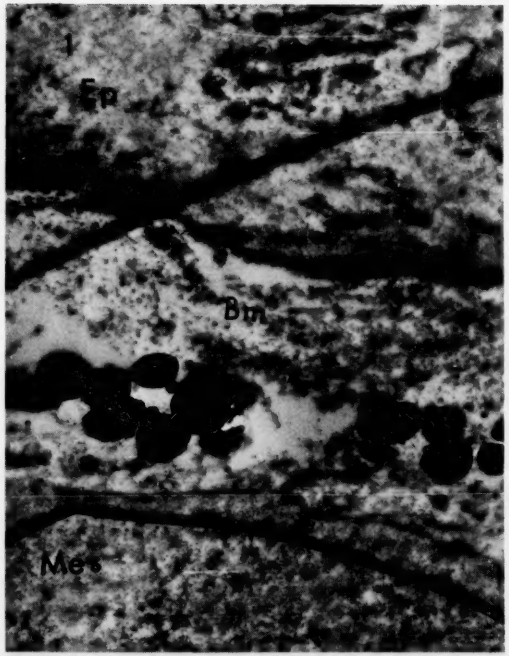

FIG. 1.—Electron micrograph of the border between the epidermis and mesenchyme in normal skin of *Amblystoma* at the stage when limb inductions appear. *Ep*, epidermis; *Mes*, mesenchyme; *Bm*, basement membrane of epidermis.

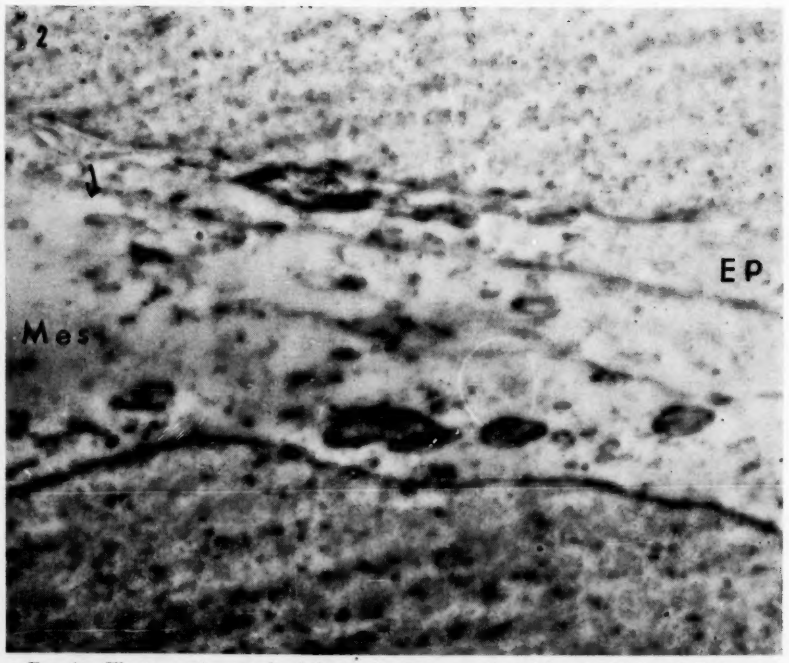

FIG. 2.—Electron micrograph of the border between the epidermis (*Ep*) and the mesenchyme (*Mes*) in an induced limb rudiment. The arrow shows a section in which the cytoplasm of the mesenchyme cell is in direct contact with the cytoplasm of the epidermal cell.

The induction of a heterotopic limb may then well be a direct result of the absence of the epidermal basement membrane at a particular spot, correctly timed, so that the competent mesodermal cells are able to establish direct contact with the epidermis. The grafts serving as limb inductors appear to be capable of influencing adversely the development of the basement membrane in adjoining sections of the skin. In the case of the nose rudiment, a zone denuded of the basement membrane is found around the opening (the nostril) by which the grafted olfactory sac communicates with the exterior. The basement membrane is also greatly reduced (even to complete disappearance) in the epidermis overlying a grafted ear vesicle. If induced limb rudiments are present, these invariably occupy the position where the deficiencies of the basement membrane are the greatest, but areas with a reduced basement membrane often reach beyond the induced limb rudiments. According to the theory proposed here, the potential limb cells of the mesoderm establish a contact with the epidermis in the denuded areas, are trapped there, and thus form the rudiment of an induced limb. It is possible that once a limb rudiment has been started, it retards further the development of the basement membrane. This suggestion may perhaps account for the development of the normal forelimbs in urodeles, while in the case of the hind limbs there may prove to be some local factors preventing the formation of the basement membrane until the hind-limb development gets under way.

It is to be hoped that some means may be found to destroy the basement membrane of the epidermis selectively. Performed at an appropriate stage and place, this should lead to supernumerary limb development and thus furnish a crucial test for the new theory. In the meantime I should like to indicate that some known phenomena appear to fall in line with the new interpretation.

1. Holtfreter[7] has reported a very peculiar transformation of fin folds into limbs. One can understand this transformation if one takes into account that the fin folds in question were situated in the area of the lateral plate mesoderm, which is presumably the source of the mesodermal limb cells, and, furthermore, that the basement membrane of the epidermis, as I have seen in my preparations, is extremely attenuated in the dorsal fin fold and disappears toward its tip. It is very likely, then, that lateral mesoderm cells may, under the circumstances, migrate into the fin fold and, finding contact with the epidermis, settle down to form a limb rudiment.

2. It has been repeatedly asserted that if the amputation surface of a limb is covered by skin (whether by natural overgrowth from the sides or deliberately, by the experimenter), regeneration is suppressed. The dermis of the skin has usually been held responsible for this effect. In the light of the present experiments it would seem that the important factor may well be the basement membrane of the epidermis covering the wound. The success of the methods proposed to renew or extend the regeneration capacity in tadpoles and adult frogs[8, 9] may depend on the effective prevention or retardation of basement-membrane development.

3. In Nassonov's experiments[10] on additional limb development after ligaturing of limbs or after implantation of pieces of cartilage under the skin of a limb, etc., the mechanism of the morphogenetic stimulus may well have been a local destruction of the epidermal basement membrane. The limb mesoderm cells coming into contact with the denuded area may then have formed a new blastema, from which stage onward the development would have followed the pathway of normal regeneration.

A study of the basement membrane of the epidermis in all similar cases is desirable and will easily show whether my suggestions are correct.

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ABNORMAL BRAIN SIZE AND FUNCTIONAL REGULATION IN FUNDULUS

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Communicated by J. S. Nicholas, August 2, 1956

Introduction.—The development of the behavior pattern has been described[1] for a group of *Fundulus heteroclitus* embryos with central nervous systems abnormal as a result of transplantation experiments that had been performed during gastrula stages. The results of that investigation showed a surprising degree of functional regulation in embryos in which supernumerary brain parts had developed in confluence with the primary brain. It was therefore concluded that the developing central nervous system can under some conditions integrate considerably more than the usual amount of material into an orderly functioning whole.

Not all the operated embryos, however, regulated completely. Since the deficits in behavior of the nonregulating embryos could not always be correlated with specific visible morphological disturbances of the brain, it has seemed desirable to attempt a quantitative study of the abnormal brains in order to ascertain whether brain size may have been a factor affecting the degree of regulation which took place in the operated embryos.

Procedure.—Paper reconstructions were made of the brains of forty-five of the fifty embryos that were discussed in detail in the earlier communication. With the aid of the camera lucida, alternate sections of the brains studied were drawn on heavy bond paper at a magnification of 130×. The reconstructions constituted by the alternate drawn sections were weighed and their weights doubled to give the total relative weights indicated for each separate embryo by the vertical light gray and dark gray bars in Figure 1. The protocol numbers of the embryos, shown along the abscissae of the graph, correspond to those in Tables 1–5 of the original communication. The asterisks designate the brains illustrated by photomicrographs in the plates of that article.

Comparable reconstructions were made of nine unoperated normal *Fundulus* brains. Since the operated embryos were fixed at stage 31, 32, or 33,[2] three unoperated normal brains were chosen from each of these stages. It was shown

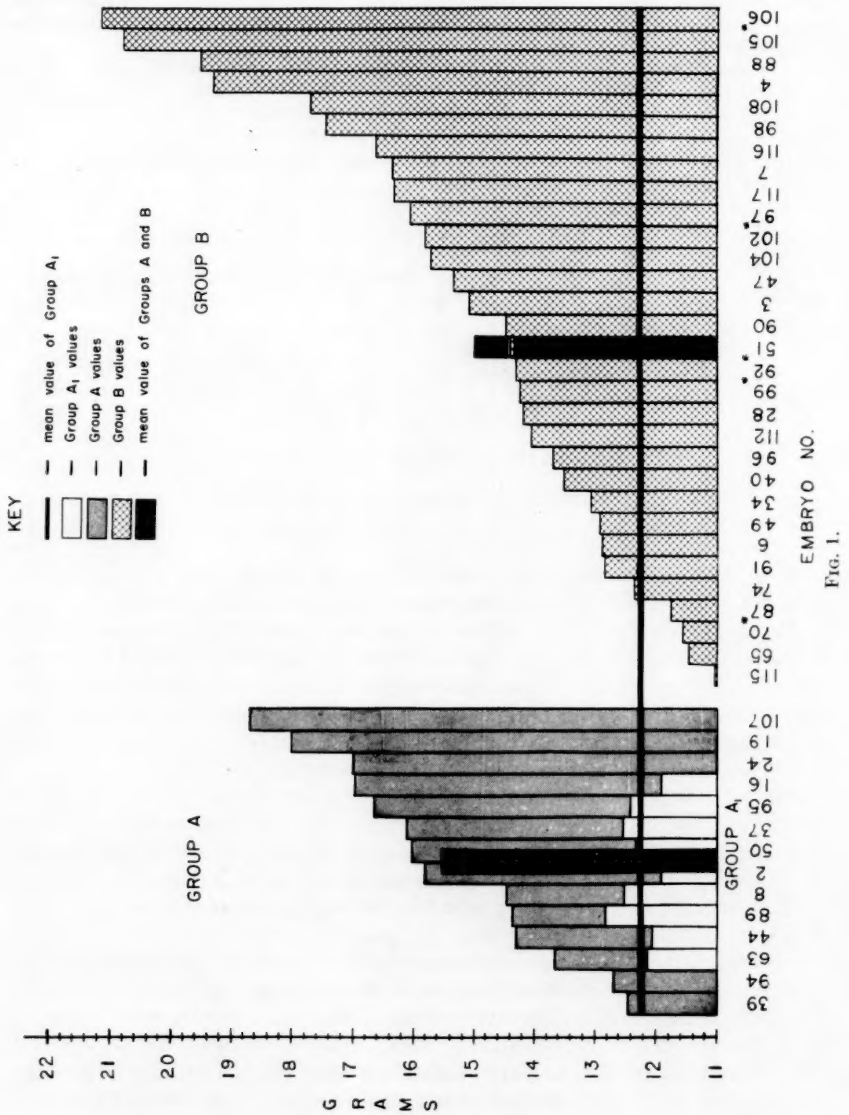

Fig. 1.

statistically by the confidence-level method[3] that the values for the set of nine unoperated normal brain models (Group A_1, indicated by white vertical bars in the graph) are so close to their mean (12.279 gm.; horizontal black line in the graph)

as to render the deviation of each value from the mean insignificant. The variance for the unoperated normal group, as calculated by the method of Snedecor,[4] is 0.098, which is also insignificant (Table 1).

Results.—The brains of the forty-five embryos of which brain reconstructions were prepared have been divided for present purposes into two categories. One group (Group A, indicated by dark gray bars in Fig. 1) is comprised of the operated embryos in which behavior was normal in the five modalities studied; in this group are also included the four embryos previously described[1] as having occasionally tilted slightly, although they otherwise exhibited normal righting reflexes. The other group (Group B in Fig. 1, indicated by light gray bars) is constituted by the embryos in which behavior was abnormal in at least one of the modalities investigated.

TABLE 1

RESULTS OF VARIANCE ANALYSIS

	VARIANCE	VARIANCE RATIOS
Group A_1 (unoperated normal)	0.098	$\frac{A+B}{N} = 50.14$
Groups A + B (operated)	4.914	$\frac{A}{N} = 36.50$
Group A (regulated)	3.577	$\frac{B}{N} = 56.98$
Group B (nonregulated)	5.584	$\frac{B}{A} = 1.56$

It is evident from the figure that the weights of the brain models of the operated embryos are, in all except four cases in Group B, greater than the mean value of the brain model weights for the unoperated normal embryos. The mean value for Group A (15.504 gm.) and that for Group B (15.030 gm.) are both significantly higher than that for the unoperated normal group (12.279 gm.). Furthermore, the variance ratios of the operated as compared with the unoperated normal group are highly significant (Table 1). The difference, however, between the means of Group A and Group B is not statistically significant, as determined by the confidence-level method. The ratio of the variance of Group B as compared to that of Group A is likewise not statistically significant (Table 1).

Conclusions.—Since the variance of the relative weights of the brains of the regulating group does not differ significantly from that for the nonregulating group, it may be concluded that the size of the brain per se, within the limits under consideration here, is not a critical factor influencing regulation of developing nervous function. These quantitative results confirm the suggestion made in the previous morphological study[1] that functional regulation can occur in embryos with brains significantly larger than the normal. They imply, as did the earlier results, that functional regulation in these operated brains has been governed by a pattern of organization which is, at least within certain limits, independent of the size and specific structure of the regulating whole.

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[3] F. E. Croxton and D. H. Cowden, *Applied General Statistics* (New York: Prentice-Hall, Inc., 1939), p. 314.

[4] G. W. Snedecor, *Calculation and Interpretation of Analysis of Variance and Covariance* (Ames, Iowa: Collegiate Press, Inc., 1934).

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